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ESTRUTURA GENÉTICA E ANÁLISE DE GARGALO POPULACIONAL EM TARTARUGA-CABEÇUDA (*Caretta caretta*) NO ATLÂNTICO SUL OCIDENTAL

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"Ninguém caminha sem aprender a caminhar, sem aprender a fazer o caminho caminhando, refazendo e retocando o sonho pelo qual se pôs a caminhar".

Paulo Freire

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RESUMO

O Atlântico Sul Ocidental (ASO) abriga importantes áreas de desova e de alimentação de tartaruga-cabeçuda (*Caretta caretta*). A maior parte dos estudos realizados na região com esta espécie são direcionados a fêmeas e juvenis e pouco se sabe sobre a ocorrência de machos nesta região, especialmente sobre padrões de uso de habitat e alimentação. O ASO também serve de área de atuação de inúmeras frotas pesqueiras, que frequentemente sobrepõem-se às áreas de alimentação de tartaruga-cabeçuda. Atualmente, as atividades pesqueiras são consideradas a principal ameaça às populações de tartarugas marinhas devido às altas taxas de captura e mortalidade associadas a esta interação. Populações que sofrem drásticas reduções no tamanho populacional estão sujeitas ao efeito de gargalo populacional, caracterizado pela perda significativa da variabilidade genética e um aumento nas taxas de endocruzamento entre os indivíduos. Este estudo teve como objetivo caracterizar geneticamente as principais áreas de desova e alimentação de tartaruga-cabeçuda no ASO, identificar a ocorrência de evento de gargalo populacional, e estimar a origem e ecologia trófica de machos desta espécie. A amostragem das colônias foi realizada nos estados de Sergipe, Bahia, Espírito Santo e Rio de Janeiro, Brasil. As áreas de alimentação foram avaliadas a partir de amostras obtidas na captura incidental de tartarugas-cabeçuda em curral de pesca, no Ceará, e no arrasto de parelha e na pesca de espinhel pelágico, no Rio Grande do Sul (RS). Além disso, foram amostradas tartarugas encalhadas ao longo do litoral sul do RS. A análise de fragmentos (~800 pb) da região controle do DNA mitocondrial revelou que as áreas de alimentação de tartarugas-cabeçuda são constituídas em maior proporção por indivíduos provenientes das colônias brasileiras. Em menor proporção, foi observado que a região costeira do estado do Ceará abriga indivíduos com haplótipos característicos de colônias do Atlântico Norte e Leste, enquanto que no sul do Brasil foram identificados indivíduos do Indo-Pacífico, principalmente entre juvenis da região oceânica. Entre os machos amostrados, 88,5% apresentaram haplótipos de colônias brasileiras, sendo que foi estimado que as áreas de desova do Espírito Santo e Rio de Janeiro são as que contribuem em maior proporção com machos nas áreas de alimentação no sul do Brasil. A análise de isótopos estáveis de carbono e nitrogênio indicou variações na alimentação e uso do habitat entre machos adultos e juvenis. Os machos adultos utilizam preferencialmente a região costeira e alimentam-se em maior proporção de invertebrados bentônicos, como o ermitão *Loxopagurus loxochelis* e o gastrópode *Buccinanops monoliferum*. Já os juvenis

apresentaram uma alta variabilidade nos valores isotópicos, revelando o uso do ambiente costeiro e do ambiente oceânico. Para estes indivíduos, tanto as presas pelágicas (por exemplo, salpas), como as bentônicas (ermitão *L. loxochelis*) destacaram-se como os itens que mais contribuem na alimentação dos juvenis. A análise de nove *loci* de microssatélites de tartarugas-cabeçuda das áreas de desova do ASO revelou uma alta diversidade genética, com uma alta frequência de alelos raros. Não foi observada diferenciação genética entre as colônias através dos índices de distância genética, nem na análise de isolamento por distância geográfica. As análises de gargalo populacional revelaram uma deficiência de alelos raros na colônia de Sergipe, mas a estimativa de excesso de heterozigosidade não revelou perda significativa de diversidade genética e indicou que as colônias avaliadas estão em equilíbrio de mutação e deriva. Os valores da razão-M, no entanto, mostraram a ocorrência de evento de gargalo compatível com um desequilíbrio demográfico nas últimas gerações. Embora as análises de gargalo populacional indiquem que a população está em equilíbrio, o indício de que a população de tartaruga-cabeçuda do ASO sofreu uma redução drástica nas gerações passadas deve ser considerado na implementação e / ou manutenção de medidas de conservação.

Palavras-chave: captura incidental, conservação marinha, DNA mitocondrial, microssatélites, modelos bayesianos de mistura.

ABSTRACT

The southwestern Atlantic Ocean (SWA) holds important nesting and foraging grounds of loggerhead sea turtles (*Caretta caretta*). Most studies at the region are focused on adult females and juveniles, and little is known about males, mainly in terms of habitat use and feeding behavior. The SWA also harbors numerous fisheries fleets that frequently overlap with loggerhead foraging grounds. Currently, fishery activities are known to be the highest threat to loggerhead turtles due to high capture and mortality rates associated with this interaction. This can lead to pronounced reductions in abundance and these populations are consequently subject to the population bottleneck effect, characterized by significative loss in genetic diversity and increase in inbreeding rates among individuals. The goals of the present study were to characterize the genetic composition of nesting and foraging grounds of loggerhead turtles from SWA, identify occurrence of populational bottleneck events, and estimate origins and trophic ecology of males of this species. Sampling of rookeries was performed in Sergipe, Bahia, Espírito Santo and Rio de Janeiro states, Brazil. Foraging grounds were assessed through loggerhead turtles caught in fish weir, in Ceará state, and pair bottom trawl and pelagic longline fisheries, in Rio Grande do Sul (RS) state. Furthermore, loggerhead turtles washed ashore were sampled along the coastline of RS. A fragment of the mitochondrial DNA control region (~800 pb) revealed that foraging aggregations are composed mainly by individuals from Brazilian rookeries. In a smaller proportion, it was observed that the coastal region of the Ceará state holds individuals with haplotypes reported in the North and East Atlantic, whereas in southern Brazil, individuals from Indian and Pacific nesting grounds were identified, mainly among early juveniles in oceanic waters. Among the sampled male loggerheads, 88.5% had haplotypes from Brazil, mainly from the Espírito Santo and Rio de Janeiro rookeries. Stable isotope analysis of carbon and nitrogen indicated variations in feeding behavior and habitat use among adults and juvenile males. Adult males preferentially use coastal areas and feed upon benthic invertebrates, such as the ermit crab *Loxopagurus loxochelis* and the gastropod *Buccinanops monoliferum*. Male juveniles showed high variability in stable isotope values, revealing the use of both neritic and oceanic habitats. These individuals, pelagic prey (salps) as well as benthic invertebrates (hermit crab) stood out as the food sources that most contributed do the diet of juveniles. Analysis of nine microsatellites *loci* of loggerhead turtles from nesting grounds in the SWA showed high genetic variability, with a high proportion of rare alleles. Genetic

differentiation was not observed between colonies through pairwise genetic distance or isolation by geographic distance. Population bottleneck analysis revealed rare allele deficiency in Sergipe rookery, but estimates of heterozygosity excess did not show significant loss in genetic variability and indicated that nesting grounds are in mutation drift equilibrium. The M-ratio values, however, showed sign of bottleneck events compatible with a demographic reduction in last generations. Although population bottleneck analyses indicate that the population is currently in equilibrium, the evidence that the loggerhead population in SWA has experienced a drastic reduction in last generations should be considered in the implementation and/or maintenance of conservation measures.

Keywords: incidental bycatch, mitochondrial DNA, marine conservation, microsatellites, Bayesian mixing models.

INTRODUÇÃO

A tartaruga-cabeçuda (*Caretta caretta*) é uma das seis espécies de tartarugas marinhas pertencente à família Cheloniidae e apresenta uma ampla distribuição geográfica, ocorrendo em águas tropicais e subtropicais de todos os oceanos (Casale e Tucker 2017). Morfologicamente, esta espécie é caracterizada por apresentar uma cabeça larga, com dois pares de escamas interorbitais e ranfoteca queratinosa robusta, e carapaça de tonalidade marrom com cinco placas laterais (Fig.1, Wyneken 2001). Não há dimorfismo sexual entre juvenis. No entanto, na fase adulta, é possível diferenciar os machos das fêmeas, pois os machos apresentam uma longa cauda com a abertura cloacal presente na extremidade posterior (Wyneken 2001).

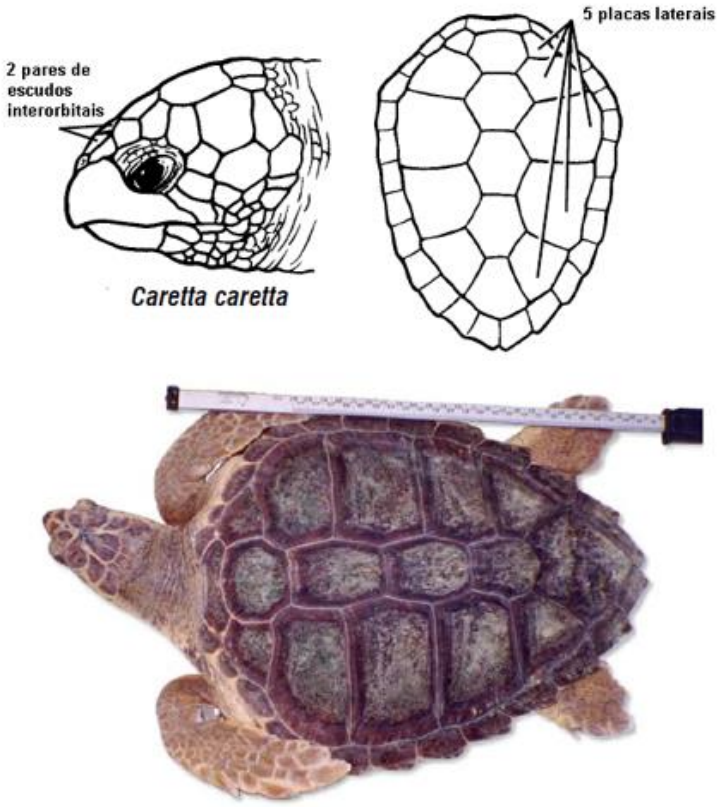

Fig. 1. Características morfológicas da tartaruga-cabeçuda (*Caretta caretta*). Modificado de Wyneken (2001).

Semelhante às outras espécies de tartarugas marinhas, a tartaruga-cabeçuda apresenta um complexo ciclo de vida, caracterizado por mudanças ontogenéticas na dieta e no uso de habitat (Fig. 2), fidelidade às áreas de desova, migrações entre as áreas de alimentação e de reprodução, e maturação sexual tardia (Bolten 2003, Jensen et al. 2013).

De maneira geral, as tartarugas-cabeçuda passam seus primeiros anos de vida em habitats oceânicos (~ 12 anos, Petitet et al. 2012), alimentando-se, oportunisticamente, de presas pelágicas (Jones e Seminoff 2013). Após este período, os juvenis passam por uma fase de recrutamento para o ambiente nerítico, onde permanecem até a fase adulta, alimentando-se preferencialmente de invertebrados bentônicos (Bolten 2003, Barros 2010, Jones e Seminoff 2013). Ao atingirem a maturidade sexual, por volta dos 30 anos de idade (Petitet et al. 2012), fêmeas e machos adultos passam a realizar migrações sazonais para as áreas de reprodução, normalmente localizadas em suas áreas natais (FitzSimmons et al. 1997, Jensen et al. 2013, Clusa et al. 2018).

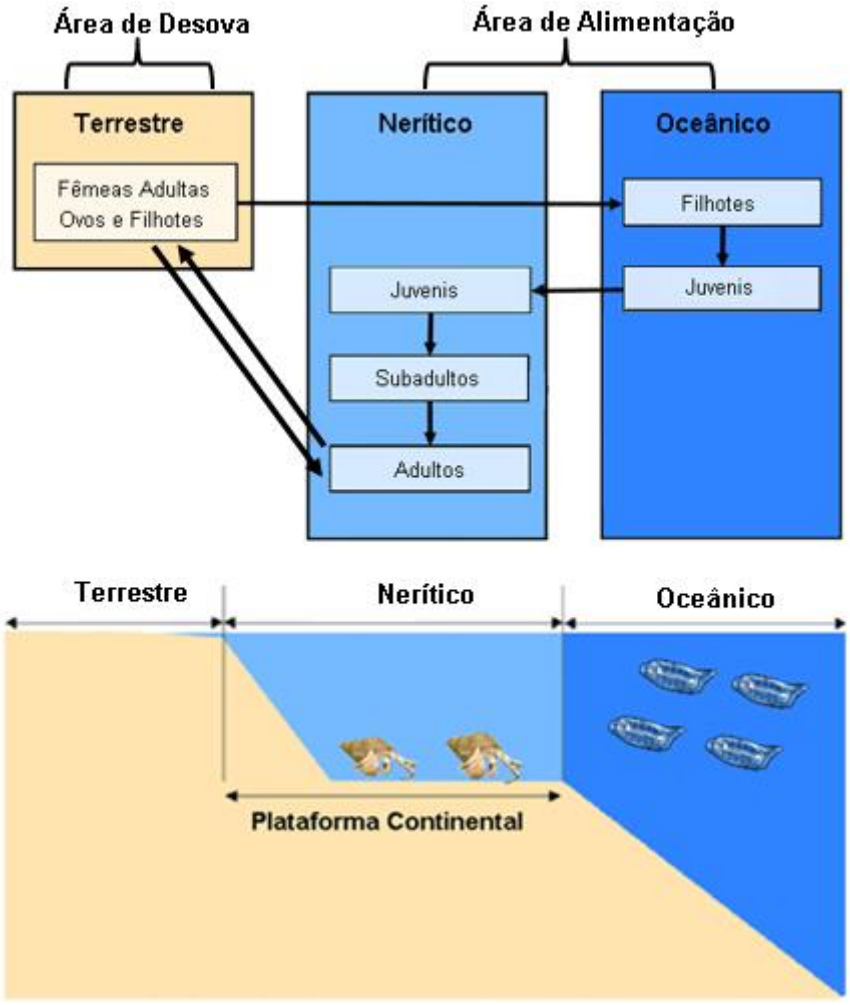

Fig. 2. Modelo ontogenético generalizado da tartaruga-cabeçuda (*Caretta caretta*), destacando os habitats e a alimentação nas diferentes fases de vida. Modificado de Figgener et al. (2019).

No entanto, estudos indicam que há variações neste modelo generalizado de história de vida, tanto no comportamento migratório como no de forrageio (Hatase et al. 2002b, Casale et al. 2007, Mansfield et al. 2009, McClellan et al. 2010, Vander Zanden et al. 2010, Zbinden et al. 2011). Os fatores que causam essas variações podem estar relacionados a mudanças sazonais na temperatura da superfície do mar (Mansfield et al. 2009, Monteiro 2017) e nas correntes marinhas (Mansfield et al. 2017), e à disponibilidade de recursos (Pajuelo et al. 2016).

Ao longo de seu ciclo de vida, as tartarugas-cabeçuda estão expostas a diferentes ameaças antrópicas, como degradação do ambiente costeiro, poluição dos oceanos e, principalmente, captura incidental em diversas artes pesqueiras (Clusa et al. 2016, Lutcavage et al. 1997, Monteiro et al. 2016, Rizzi et al. 2019). Por esta razão, embora o tamanho de inúmeras populações de tartaruga-cabeçuda atualmente seja considerado estável ou crescente, esta espécie é classificada como "Vulnerável" na Lista Vermelha da IUCN e esta tendência de crescimento está associada a planos de conservação em áreas de desova e alimentação ao redor do mundo (Rees et al. 2016, Casale e Tucker 2017). Neste contexto, compreender os padrões de uso de habitat e de recursos e a conectividade entre as áreas de alimentação e de desova é essencial para o desenvolvimento e/ou manutenção de medidas de manejo adequadas para a conservação da espécie (Rees et al. 2016).

A análise de isótopos estáveis (AIE) é um método amplamente aplicado em tartarugas marinhas para identificar fontes alimentares e relações tróficas (Dodge et al. 2011, Jones e Seminoff 2013), uso de habitat (Reich et al. 2010, Petitet e Bugoni 2017, Raposo et al. 2019) e mudanças ontogenéticas (Snover et al. 2010, Turner-Tomaszewicz et al. 2016). Isótopos estáveis de nitrogênio ($^{15}N/^{14}N$ – $\delta^{15}N$) são utilizados para avaliar o nível trófico de um consumidor (DeNiro e Epstein 1981, Minagawa e Wada 1984), enquanto que os valores isotópicos de carbono ($^{13}C/^{12}C$ – $\delta^{13}C$) permitem diferenciar fontes alimentares e distinguir o uso de diferentes habitats marinhos (e.g., nerítico *vs.* oceânico, Fry 2006). Um aspecto importante na AIE é a escolha do tecido a ser analisado, pois apresentam composições e taxas de renovação distintas (Fry 2006, Perkins et al. 2013). Os valores de isótopos estáveis dos tecidos de um consumidor reflete a composição isotópica de suas presas em uma escala de tempo que depende da taxa de renovação do tecido (Peterson e Fry 1987, Fry 2006). Em tartarugas-cabeçuda, por exemplo, a pele fornece valores isotópicos que correspondem a um período de tempo de ~45 dias (Reich

et al. 2008). Por essa razão, é considerado um tecido adequado para identificar padrões alimentares recentes (Reich et al. 2008, Petitet e Bugoni 2017).

Nas últimas décadas, a AIE e a telemetria revelaram distintos padrões migratórios, de uso de habitat e estratégias de forrageio de tartarugas-cabeçuda (Hawkes et al. 2006, McClellan et al. 2010, Pajuelo et al. 2012, Pajuelo et al. 2016, Monteiro 2017). Esses estudos observaram que as tartarugas-cabeçuda podem apresentar dicotomia no uso de habitat, em alguns casos relacionada ao tamanho corporal das tartarugas. No caso das fêmeas adultas no Pacífico Ocidental e leste do Oceano Atlântico, foi observado que tartarugas-cabeçuda de tamanho corporal maiores preferencialmente ocupavam habitats neríticos, enquanto que as menores ocorriam preferencialmente em habitats oceânicos (Hatase et al. 2002b, Hawkes et al. 2006). Para machos adultos do noroeste do Pacífico, também foi relatado um padrão semelhante ao das fêmeas (Saito et al. 2015, Hatase et al. 2002a). No Mar Mediterrâneo, no entanto, embora polimorfismos no uso do habitat tenham sido observados em machos adultos, esta variabilidade não foi associada ao tamanho dos indivíduos (Schofield et al. 2010). Além disso, no noroeste e sudeste do Atlântico (Arendt et al. 2012, Varo-Cruz et al. 2013) e no Mar Mediterrâneo (Schofield et al. 2010, Casale et al. 2013) foi relatado que machos adultos podem apresentar alta fidelidade e comportamento de residência em áreas de reprodução e alimentação, e que algum grau de especialização individual pode ser observado devido à disponibilidade de recursos (Pajuelo et al. 2016). Apesar do crescente conhecimento sobre machos de tartaruga-cabeçuda em diferentes bacias oceânicas, são escassas as informações ecológicas a respeito deste grupo no Atlântico Sul Ocidental (ASO).

O ASO abriga importantes áreas de desova, alimentação e desenvolvimento de tartaruga-cabeçuda (Marcovaldi e Marcovaldi 1999, Vélez-Rubio et al. 2013, Carman et al. 2016, Monteiro et al. 2016). Na costa brasileira, observa-se uma das maiores concentrações de desova do Oceano Atlântico (7000 a 8000 ninhos/ano, Marcovaldi et al. 2018), com colônias distribuídas entre os estados de Sergipe (região nordeste) e Rio de Janeiro (região sudeste) (Fig. 3, Marcovaldi e Chaloupka 2007). Dados de rastreamento por satélite de fêmeas adultas provenientes de áreas de desova no estado da Bahia revelaram uma alta fidelidade a áreas de forrageio neríticas durante e após o período reprodutivo (Marcovaldi et al. 2010). Os deslocamentos entre as áreas de desova e alimentação ocorreram ao longo da plataforma continental, e o estado do Ceará destacou-se como a principal região de forrageio para as fêmeas de tartaruga-cabeçuda (Marcovaldi

et al. 2010). Além disso, estudos de monitoramento da pesca de curral nesta região mostraram que o ambiente costeiro do Ceará também é ocupado por grandes juvenis desta espécie (Lima et al. 2013). Importantes áreas de alimentação de tartaruga-cabeçuda também já foram identificadas no sul do Brasil: enquanto a região costeira é ocupada principalmente por adultos e grandes juvenis, a região oceânica é utilizada prioritariamente por pequenos juvenis (Bugoni et al. 2003, Barros et al. 2010, Petitet et al. 2012, Monteiro et al. 2016). Análises do conteúdo gastrointestinal de tartarugas-cabeçuda indicaram a ingestão de cerca de 45 tipos de presas por esta espécie na região. No entanto, foi relatado que no ambiente oceânico as tartarugas-cabeçuda consomem predominantemente salpas e pirossomos, enquanto que em áreas neríticas predam principalmente ermitões e gastrópodes (Bugoni et al. 2003, Barros 2010).

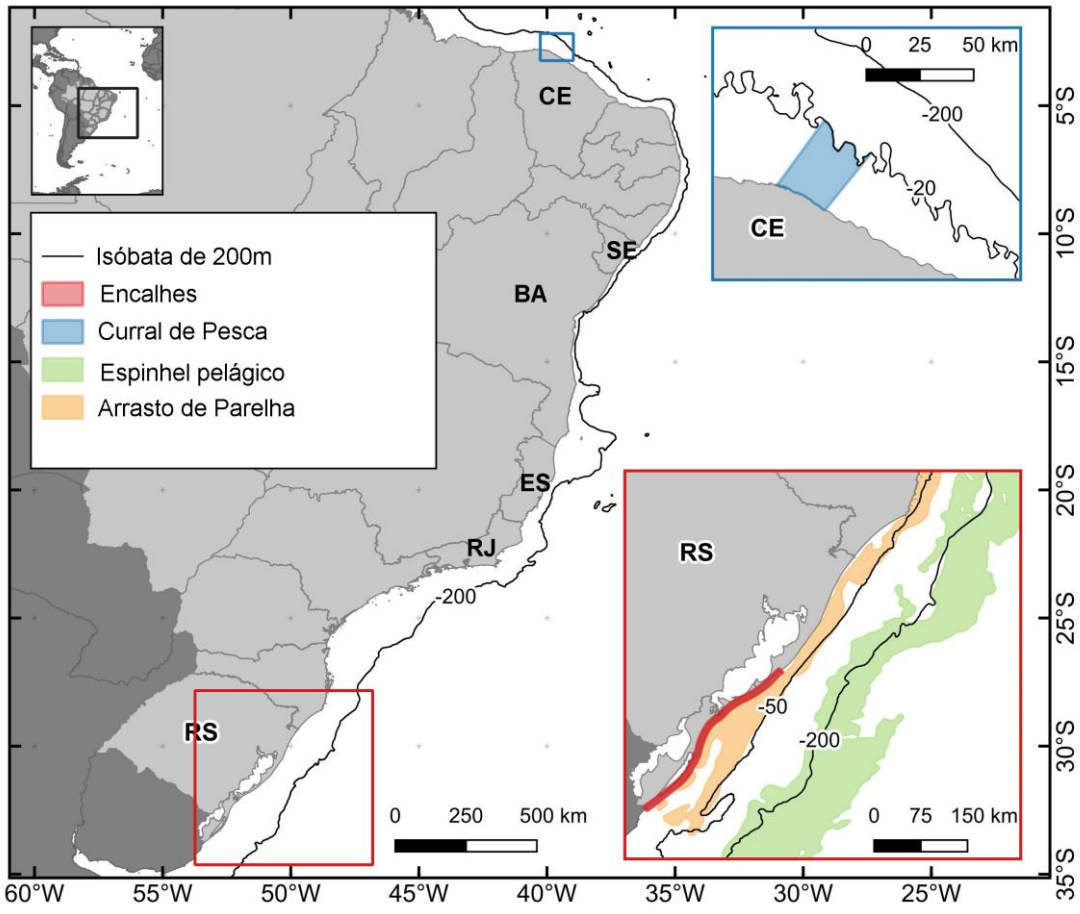

Fig. 3. Áreas de desova e de alimentação de tartaruga-cabeçuda (*Caretta caretta*) no Atlântico Sul Ocidental. As áreas ampliadas indicam as regiões de amostragem de tartarugas-cabeçuda capturadas na pesca e encalhadas no presente estudo. Abreviações: CE = Ceará, SE = Sergipe, BA = Bahia, ES = Espírito Santo, RJ = Rio de Janeiro, RS = Rio Grande do Sul.

Assim como no restante do mundo, no ASO a tartaruga-cabeçuda apresenta um longo histórico de ameaças. Antes da década de 80, a maior parte dos ovos eram coletados e as fêmeas eram abatidas para alimentação (Silva et al. 2016). A partir deste período, com a criação e implementação do Projeto TAMAR nas principais áreas de desova, estratégias de manejo começaram a contribuir para a recuperação das populações brasileiras, com grande aumento na abundância das fêmeas em desova e no número de ninhos por temporada (Marcovaldi e Chaloupka 2007, Marcovaldi et al. 2018). No entanto, outras atividades humanas têm afetado consideravelmente a sobrevivência das tartarugas marinhas. Atualmente, a interação das tartarugas-cabeçuda com a pesca é considerada a principal causa de mortalidade destes animais (Wallace et al. 2013). Nas últimas décadas, elevadas taxas de captura incidental foram registradas na região costeira e oceânica do ASO, principalmente pela pesca de emalhe (Fiedler et al. 2012), curral de pesca (Lima et al. 2013), arrasto de parelha (Monteiro et al. 2013, Monteiro 2017), e espinhel pelágico (Giffoni et al. 2014, Sales et al. 2008). Para esta última, por exemplo, foi estimado que 6594 tartarugas-cabeçuda foram capturadas entre 1998 e 2010 em águas brasileiras e águas internacionais adjacentes (Giffoni et al. 2014). Contudo, informações sobre os efeitos destas atividades na estrutura demográfica e genética das populações ainda são escassas.

Uma das consequências da redução significativa na abundância de uma população é a ocorrência do efeito de gargalo populacional, que resulta na perda da diversidade genética e do potencial evolutivo, aumento de endocruzamentos, e deriva genética (Frankham et al. 1999). Considerando que populações que recentemente passaram por eventos de gargalo apresentam deficiência de alelos raros, é possível detectar eventos deste tipo através da análise da distribuição de frequências alélicas e do excesso de heterozigosidade (Cornuet e Luikart 1996). Análises de DNA nuclear (nDNA) revelaram um claro sinal de evento de gargalo em seis das treze populações de tartaruga-oliva (*Lepidochelys olivacea*) que desovam no México, revelando um padrão consistente com o desequilíbrio demográfico causado por décadas de intensa exploração comercial (Rodríguez-Zárate et al. 2013). Em contrapartida, apesar da intensa caça de fêmeas e machos, da coleta de ovos e da captura incidental na pesca de tartarugas-cabeçuda nas ilhas de Cabo Verde, na África, não foi detectado sinal de evento de gargalo nestas populações (Monzón-Argüello et al. 2010). Estes resultados contrastam com a alta taxa de mortalidade na região e indicam que o cenário atual ainda não comprometeu a

diversidade genética da população. No Brasil, os dados disponíveis sobre a abundância populacional da tartaruga-cabeçuda são escassos e baseados prioritariamente no número de fêmeas desovantes a partir da década de 80, o que inviabiliza uma avaliação adequada sobre a potencial ocorrência de um declínio demográfico significativo associado aos inúmeros impactos na região (Santos et al. 2011). No entanto, ao longo da costa brasileira há uma elevada frequência de híbridos de tartarugas marinhas; análises genéticas indicam que este fenômeno é recente (~40 anos) e que pode representar uma estratégia reprodutiva diante da falta de parceiros da mesma espécie (Vilaça et al. 2012). Embora a hibridização seja considerada natural na história evolutiva dos organismos, o declínio abrupto do tamanho das populações constitui uma das possíveis causas para a ocorrência deste evento, especialmente quando observado em altas frequências em uma determinada região (Allendorf et al. 2001).

Técnicas moleculares têm desempenhado um importante papel no estudo da ecologia de tartarugas marinhas (Jensen et al. 2013) através da caracterização genética das populações e descrição de unidades de manejo (MUs - *sensu* Moritz 1994) demograficamente independentes (Jensen et al. 2013, Shamblin et al. 2014), estimativa do fluxo gênico (Stiebens et al. 2013) e conectividade entre áreas de alimentação e reprodução (Jensen et al. 2013, Shamblin et al. 2014, Tolve et al. 2018). As MUs são definidas como populações com significativa diferenciação genética (Moritz 1994), e uma definição adequada destas unidades é fundamental para estabelecer medidas de conservação. Apesar da alta capacidade migratória das tartarugas marinhas, a dispersão de genes (fluxo gênico) entre as populações não reflete este comportamento. Ao contrário, populações de tartarugas marinhas apresentam uma significativa estruturação da linhagem materna, observada através do DNA mitocondrial (mtDNA), causada pela fidelidade das fêmeas às áreas de desova em que nasceram (filopatria natal). Em alguns casos, esta estruturação populacional também pode ser detectada no nDNA, padrão atribuído ao baixo fluxo de genes paternos (Clusa et al. 2018).

Recentemente, a relação filogeográfica das principais áreas de desova de tartaruga-cabeçuda do Mar Mediterrâneo e dos Oceanos Atlântico e Índico foi avaliada através da análise de fragmentos do mtDNA, e delineou ao menos 18 MUs entre as bacias oceanográficas (Shamblin et al. 2014). No Brasil, observou-se que as colônias são geneticamente distintas das demais populações do mundo, apresentando um perfil genético exclusivo, com a ocorrência de haplótipos endêmicos da região (Reis et al. 2010,

Shamblin et al. 2014). Ademais, as áreas de desova no Brasil foram sugeridas como três MUs distintas no ASO: (1) Sergipe e Bahia, (2) Espírito Santo e (3) Rio de Janeiro (Shamblin et al. 2014). No entanto, essa diferenciação genética entre as localidades não está bem definida, e um aumento do número de indivíduos analisados, bem como a utilização de outros marcadores moleculares (e.g., microssatélites), foi sugerido para melhor compreender a estrutura populacional no ASO (Shamblin et al. 2014).

A diferenciação genética que existe entre áreas reprodutivas de tartarugas marinhas permite estimar as origens natais de tartarugas em áreas de alimentação, que são geralmente compostas por agregações de indivíduos provenientes de múltiplas áreas de desova, denominadas "estoques mistos" (Jensen et al. 2013, Rees et al. 2017, Tolve et al. 2018). Em um estudo prévio envolvendo 125 juvenis de tartarugas-cabeçuda capturados no espinhel pelágico na região oceânica do sul do Brasil, foi demonstrado que a maior parte dos indivíduos que compõem a área de alimentação provem das áreas de desova brasileiras (47,2%) e, em menor proporção, de colônias do Atlântico Norte/Mediterrâneo (25,6%) e Austrália (12%) (Reis et al. 2010, Shamblin et al. 2014). Em contrapartida, foi observado que as áreas de alimentação de *C. caretta* em águas costeiras do Uruguai e Argentina são compostas exclusivamente por indivíduos originários de populações brasileiras (Caraccio et al. 2008, Prosdocimi et al. 2015). No entanto, informações sobre a composição dos estoques mistos e a estrutura das principais áreas de alimentação de tartaruga-cabeçuda no Brasil são escassas, especialmente aquelas que se sobrepõem às áreas de pesca em habitats neríticos.

HIPÓTESES

Considerando o exposto acima, as hipóteses testadas no presente estudo foram: (1) os estoques mistos que utilizam habitats costeiros são geneticamente distintos dos que habitam áreas oceânicas; (2) machos de tartaruga-cabeçuda apresentam variações no uso de habitat relacionadas à fase de vida; e (3) as colônias brasileiras apresentam sinal de evento de gargalo populacional como reflexo de um desequilíbrio demográfico significativo nas últimas décadas.

OBJETIVOS

O objetivo geral desta tese foi estimar a origem e estrutura genética, identificar padrões de dispersão e uso de habitat, e verificar a ocorrência de evento de gargalo populacional em tartarugas-cabeçuda do ASO. Os objetivos específicos foram: (i) estimar a contribuição das múltiplas colônias de tartaruga-cabeçuda na composição das áreas de alimentação costeiras e oceânicas no Brasil; (ii) estimar a origem e padrões de uso de habitat e alimentação de machos de tartaruga-cabeçuda que ocorrem no sul do Brasil; (iii) e verificar a ocorrência de evento de gargalo populacional nas colônias do ASO. Estes objetivos foram explorados em três capítulos: Capítulo 1 - "Genetic structure and origins of loggerhead sea turtle foraging aggregations in the southwestern Atlantic Ocean: fisheries interactions and conservation implications"; Capítulo 2 - "Origin and foraging ecology of male loggerhead sea turtles from southern Brazil revealed by genetic and stable isotope analysis"; e Capítulo 3 - "Population structure and bottleneck analyses of loggerhead sea turtles *Caretta caretta* in the southwestern Atlantic Ocean".

MATERIAL E MÉTODOS

Amostragem

Para o Capítulo 1, amostras de pele e/ou músculo foram coletadas de tartarugas capturadas no curral de pesca (n = 53), no estado do Ceará, e no arrasto de parelha (n = 54) e na pesca de espinhel pelágico (n = 181), no Rio Grande do Sul, representando áreas de alimentação do nordeste e sul do Brasil (Fig. 3). As amostragens ocorreram entre maio de 2005 e maio de 2016 no curral de pesca, de dezembro de 2013 a abril de 2017 no arrasto de parelha, e de novembro de 2005 a junho de 2017 no espinhel pelágico. Além disso, foram coletadas amostras de tecido de tartarugas-cabeçuda encalhadas (n = 119) ao longo da costa sul do Rio Grande do Sul, entre a Lagoa do Peixe (31°21'S; 051°05 'W) e o Arroio Chuí (33°44' S, 53°22' W), de outubro de 2013 a dezembro de 2017. Entre os espécimes encalhados, foram coletadas amostras adicionais de pele (n = 26) de machos para a AIE de carbono e nitrogênio (Capítulo 2). Para o capítulo 3, foram utilizadas amostras de pele obtidas de fêmeas de áreas de desova em Sergipe (n = 8), Bahia (n = 21), Espírito Santo (n = 19), e Rio de Janeiro (n = 31), coletadas entre janeiro de 2004 e janeiro de 2018 (Fig. 3). Para este capítulo também foram amostrados 21 filhotes natimortos no estado da Bahia, entre novembro de 2017 e janeiro de 2018. Em espécimes

juvenis e adultos amostrados, o comprimento curvo da carapaça (CCC) foi medido com uma fita métrica flexível (± 0,1 cm) conforme Bolten (1999). Nos natimortos, foi medido o comprimento retilíneo da carapaça (CRC) com o auxílio de um paquímetro (± 0,1 mm). As amostras de tecido coletadas para análises genéticas foram obtidas com um bisturi ou *punch* de biópsia estéreis, armazenadas em cloreto de sódio (NaCl), etanol absoluto ou solução de DMSO, e congeladas a -20°C até o processamento em laboratório. Todas as amostras de espécimes de tartaruga-cabeçuda foram coletadas em programas de monitoramento realizados pelo Núcleo de Educação e Monitoramento Ambiental - NEMA e pelo Projeto TAMAR.

Para a AIE (Capítulo 2), amostras de potenciais itens alimentares de tartaruga-cabeçuda no ambiente costeiro e oceânico também foram coletadas (Bugoni 2003, Barros 2010, Jones e Seminoff 2013). Estes itens incluíram os seguintes grupos taxonômicos: água-viva *Lychnorhiza lucerna*, o gastrópode *Buccinanops monoliferum*, o cefalópode *Dorytheuthis plei*, os ermitões *Dardanus insignis* e *Loxopagurus loxochelis*, anêmonas (Class Anthozoa, Order Actinaria) associadas às conchas dos ermitões, o caranguejo-aranha *Libinia spinosa*, salpas (Classe Thaliacea, Ordem Salpida, Família Salpidae), corvina *Micropogonias furnieri*, maria-luísa *Paralonchurus brasiliensis*, e peixe-espada *Trichiurus lepturus*. As amostras de crustáceos, anêmonas, cefalópodes e peixes foram obtidas através de frotas de pesca comercial que atuam sobre a plataforma continental do Rio Grande do Sul. As amostras de salpas foram coletadas na quebra da plataforma e talude, entre 550 e 3000 m de profundidade, em cruzeiros oceanográficos realizados pela Universidade Federal do Rio Grande - FURG. As amostras de águas-vivas e gastrópodes foram coletadas durante monitoramentos de praia ao longo do litoral sul do Rio Grande do Sul. Todas as amostras obtidas para AIE foram armazenadas em sacos plásticos e congeladas a -20°C até o processamento em laboratório.

Análises genéticas

Para as análises genéticas, o DNA genômico foi extraído de cada uma das amostras com kit de extração PureLink™ Genomic DNA Kit. A concentração e pureza do produto da extração foram verificados no espectrofotômetro de raio UV/Vis Biodrop Duo®.

Análise do mtDNA

Nos Capítulos 1 e 2, fragmentos de ~800 pares de base (pb) da região controle do mtDNA foram amplificados através de Reação em Cadeia da Polimerase (Polymerase Chain Reaction – PCR) utilizando os iniciadores LCM15382 (5- GCT TAA CCC TAA AGC ATT GG -3') e H950 (5- GTC TCG GAT TTA GGG GTT TG -3′) (Abreu-Grobois et al. 2006). As reações de PCR continham de 20 a 50 ng de DNA genômico, 5U de Platinum Taq Polymerase ou Recombinant Taq Polymerase, 0,2 μM de cada iniciador, 0,4 mM de dNTPs, 1× Tampão de PCR e 0,1 mM de $MgCl_2$. Controles negativos foram incluídos para detectar possíveis contaminações durante o processo de amplificação. As PCRs foram realizadas no termociclador Applied Biosystems Veriti 96-well sob as seguintes condições: desnaturação inicial de 5 min a 94°C; 36 ciclos de 30 s a 94ºC, 30 s a 50°C, 1 min a 72°C; e uma extensão final de 10 min a 72°C. Os produtos de PCR obtidos foram purificados com PureLink^{TM} Quick Gel Extraction and Purification Combo Kit e sequenciados na direção iniciador 3' e iniciador 5' em sequenciador ABI PRISM 3730XL Analyzer (Macrogen Inc).

As sequências obtidas foram editadas e alinhadas através do BioEdit 7.0.9 (Hall 1999) e classificadas de acordo com haplótipos previamente descritos e disponíveis nos bancos de dados Archie Carr Center for Sea Turtle Research database (http://accstr.ufl.edu/) e GenBank (http://ncbi.nlm.nih.gov). As diversidades haplotípica (h) e nucleotídica (π) (Nei 1987) foram calculadas com o programa DNAsp 5.10 (Rozas et al. 2003). Há poucas informações disponíveis acerca de índices de diversidade baseados em longas sequências de mtDNA de tartaruga-cabeçuda, o que dificulta a comparação com estudos realizados em outras áreas de alimentação do Atlântico. Por esta razão, os índices de diversidade foram calculados tanto para o fragmento curto (380 pb) quanto para o fragmento longo (~800 pb) do mtDNA.

A diferenciação genética entre as áreas amostradas no Capítulo 1 foi verificada no ARLEQUIN v. 3.1 (Excoffier et al. 2005) com base nos índices de fixação F_{st} e ϕ_{st}, utilizando apenas a frequência haplotípica e o modelo de substituição nucleotídica Tamura-Nei, respectivamente. O modelo de substituição nucleotídica mais adequado para estimar ϕ_{st} foi definido através do jModelTest 2.1.10 (Darriba et al. 2012). O grau de diferenciação genética baseado nos valores de F_{st} e ϕ_{st} foi considerado baixo, moderado, alto ou muito alto quando os valores variaram entre 0-0,05; 0,05-0,15; 0,15-0,25; e >0,25, respectivamente (Wright 1978). Uma rede haplotípica foi gerada pelo PopArt v1.7

(Bandelt et al. 1999) para ilustrar a frequência e a relação entre os haplótipos das áreas amostradas.

Para estimar a origem das tartarugas-cabeçudas amostradas em áreas de alimentação do ASO, foram realizadas Análises Bayesianas de Estoques Mistos (MSA-Mixed Stock Analysis, Pella e Masuda 2001) "muitas-a-muitas" (Capítulo 1) e "muitas-a-uma" (Capítulo 2). A MSA foi aplicada no software R versão 3.4.2 (R Core Team 2017) através do pacote "mixstock", o qual estima a probabilidade de contribuição de populações-fonte (i.e., áreas de desova) para uma ou mais agregações de tartarugas marinhas em áreas de alimentação (Bolker et al. 2007). Com base na frequência haplotípica observada nas áreas amostradas, no Capítulo 1, a matriz base do MSA foi composta pelas frequências haplotípicas descritas nas áreas de desova de tartaruga-cabeçuda no Mar Mediterrâneo, Oceanos Atlântico, Índico e Pacífico (Boyle et al. 2009, Shamblin et al. 2014, Matsuzawa et al. 2016). No Capítulo 2, a matriz base foi composta pela frequência de haplótipos descritas para as áreas de desova do ASO. Cadeias de Markov Monte Carlo (MCMC) foram aplicadas para obter as distribuições de probabilidade das contribuições dos estoques, integrando os dados de verossimilhança com uma *priori* não-informativa, resultando em distribuições de probabilidade com um intervalo de credibilidade de 95% (CrI 95%). O MCMC foi aplicado através de quatro cadeias de 120000 interações cada, com o descarte inicial das primeiras 20000 interações (Capítulo 1), e 20000 interações com descarte das 10000 interações iniciais (Capítulo 2). O fator de redução Gelman-Rubin foi utilizado para verificar a convergência das cadeias através da comparação das variâncias entre elas. Valores abaixo de 1,2 para todos os parâmetros indicam que a convergência do modelo foi atingida e que as estimativas geradas são confiáveis (Bolker et al. 2007).

Análise de microssatélites

No Capítulo 3, nove *loci* de microssatélites previamente descritos foram amplificados: Cc117, Cm72 e Cm84 (FitzSimmons et al. 1995); Cc7 e Cc141 (Bowen et al. 2005); Ccar176 (Carreras e al. 2007); Cc17, Cc25 e Cc28 (Monzón-Argüello et al. 2008). Um primer de cada *locus* foi marcado com uma fluorescência 6-FAM ou HEX, incorporada através da cauda M13 (Schuelke 2000). As PCRs foram realizadas contendo 20 a 50 ng de DNA genômico, 1,5U de Platinum Taq Polymerase, 0,1 μM do iniciador 3', 0,2 μM do iniciador 5', 0,2 μM da fluorescência, 0, 4 mM de dNTPs, 10× de tampão para

PCR e 2,5 mM $MgCl_2$. Controles negativos foram incluídos para detectar possíveis contaminações durante o processo de amplificação. Para os *loci* Cc117, Cm72, Cm84, Cc7, Cc141, as reações de PCR foram conduzidas utilizando as seguintes condições de temperatura: desnaturação inicial de 5 min a 94°C; 30 ciclos de 30 s a 94°C, 1 min a 53ºC, 45 s a 72°C; e uma extensão final de 10 min a 72°C. Para os *loci* Cc17, Cc25, Cc28 e Ccar176, as reações ocorreram utilizando um ciclo com desnaturação inicial de 5 min a 94°C; 20 ciclos de 30 s a 94°C, 30 s a 61°C (56°C para o *locus* Ccar176), 30 s a 72°C; 15 ciclos de 30 s a 94°C, 45 s a 53°C, 45 s a 72°C; e uma extensão final de 10 min a72°C. Os fragmentos obtidos foram analisados através do sequenciador ABI 3730XL com um marcador interno padrão de 400HD. Cerca de 10% das amostras foram genotipadas duas vezes independentemente para todos os *loci* para estimar a taxa de erro (Bonin et al. 2004).

O tamanho dos alelos foi definido com o Peak Scanner™ v.2 (Applied Biosystems). A presença de alelos nulos ou de erros na genotipagem foram avaliados através do MICRO-CHECKER (Oosterhout et al. 2003). Desvios do equilíbrio de Hardy-Weinberg e desequilíbrios de ligação entre pares de *loci* foram estimados pelo Genepop v. 4.5 (Rousset 2008). A riqueza alélica foi estimada no FSTAT 2.9.3 (Goudet 2001). A heterozigosidade média observada (H_o) e esperada (H_e), o número de alelos para cada *locus*, o índice de endocruzamento (F_{is}) e a distância genética entre as colônias (F_{st}) foram calculadas utilizando o ARLEQUIN 3.1 (Excoffier et al. 2005). A distância genética D_{st} também foi estimada utilizando o POPTREEW (Takezaki et al. 2014). Um teste de Mantel foi aplicado no R 3.4.2 (R Core Team 2017) para identificar a ocorrência de isolamento por distância, através da correlação entre distâncias genéticas e geográficas das áreas de desova amostradas. Além disso, foi aplicado o método Bayesiano de agrupamento para estimar o número mais provável de populações (K) na área de estudo através do STRUCTURE 2.3.4, assumindo como priori informativa que cada localidade representa uma unidade populacional distinta (Pritchard et al. 2000). O parâmetro K variou de 1 a 8; para cada K, 20 simulações e 100000 interações de MCMC com um descarte inicial de 10000 interações. O melhor K foi estimado com a média da verossimilhança das simulações e a estatística ΔK *ad hoc* no STRUCTURE HARVESTER (Evanno et al. 2005). As 20 simulações do melhor K foram combinadas no CLUMPP v.1.1.2 (Jakobsson e Rosenberg 2007) e o resultado gráfico final foi gerado no DISTRUCT v. 1.1 (Rosenberg, 2004).

A ocorrência de evento de gargalo populacional foi verificada através de três métodos distintos: análise gráfica da frequência de alelos raros na população (Luikart et al. 1998), excesso de heterozigosidade (Cornuet e Luikart 1996) e razão-*M* (Garza e Williamson 2001). O primeiro método consiste na plotagem gráfica da distribuição de frequências alélicas: gráficos cuja proporção de alelos apresentam um formato em L indicam que a população está em equilíbrio de mutação e deriva, enquanto que distribuições assimétricas indicam evento de gargalo recente (Luikart et al. 1998). O segundo método consiste na estimativa do excesso de heterozigosidade através do teste de Wilcoxon, aplicado no BOTTLENECK v.1.2.02 (Piry et al. 1999) com base no modelo de mutação de duas fases (two-phase mutation model- TPM), com a variância fixada em 22 e a proporção de mutações em 57% (Peery et al. 2012). O terceiro método consiste na razão entre o número total de alelos e a variação do tamanho do alelo (razão-M, Garza e Williamson 2001). Este método assume que para matrizes de alelos com mais de sete *loci*, valores de razão-M abaixo de 0,68 indicam que a população foi reduzida abruptamente ocasionando o evento de gargalo populacional (Garza e Williamson 2001). As análises de diversidade genética e de evento de gargalo populacional foram efetuadas em dois níveis: (1) com todas colônias do ASO agrupadas e (2) para cada área separadamente.

Isótopos estáveis

Processamento de amostras

As amostras de pele dos machos de tartaruga-cabeçuda e das potenciais presas da dieta foram lavadas com água destilada e secas em estufa a 60°C por 48 a 72 h até atingir uma massa constante. Posteriormente, pulverizadas com almofariz e pistilo. Cerca de 0,7 mg de cada amostra foi aliquotada e armazenada individualmente em cápsulas de estanho de 4 × 6 mm. Os tecidos das presas processados para a AIE foram: músculo dos peixes e crustáceos, o músculo longitudinal da coluna das anêmonas, a mesoglea das águas-vivas, o manto dos gastrópodes e cefalópodes e o organismo inteiro, no caso das salpas.

Em geral, tecidos que exibem razões C:N maiores que 3,5 contém lipídios capazes de alterar os valores de δ^{13}C (Post et al. 2007). Nesses casos, a extração de lipídios ou a normalização matemática é necessária (Post et al. 2007, Logan et al. 2008, Petitet e Bugoni 2017). De acordo com estudos anteriores desenvolvidos com tartaruga-verde (*Chelonia mydas*), a razão C:N da pele de tartarugas marinhas apresenta valores abaixo

de 3,5 e o conteúdo lipídico deste tecido não altera significativamente os valores de $\delta^{13}C$ (Vander Zanden et al. 2012, Bergamo et al. 2016). Por esta razão, a maioria das amostras de pele de machos de tartaruga-cabeçuda foram analisadas sem extrair lipídios do tecido. No entanto, as amostras de seis machos apresentaram razão C:N superiores a 3,5, indicando alto conteúdo lipídico. Por esta razão, os lipídios destas amostras de pele foram extraídos usando Soxhlet com solução 2:1 de clorofórmio:metanol por 6 h (Medeiros et al. 2015), e reanalisadas. Em relação às amostras de presas, somente as anêmonas apresentaram evidência de alto conteúdo lipídico. Neste caso, os valores isotópicos de carbono foram normalizados com base na equação proposta por D'Ambra et al. (2014).

A AIE das amostras de pele de machos de tartaruga-cabeçuda, de águas-vivas, caranguejo-aranha e gastrópodes foram analisadas com o espectrômetro de massa de razão isotópica de fluxo contínuo no Centro de Isótopos Estáveis da Universidade do Novo México (UNM–CSI). As amostras restantes foram analisadas no Laboratório de Isótopos Estáveis, na Universidade de Washington (SICL–WSU). Os valores isotópicos são expressos com a notação δ, em partes por mil (‰), em relação a padrões internacionais (Vienna Pee Dee Belemnite limestone para carbono e nitrogênio atmosférico para nitrogênio, de acordo com a seguinte equação (Bond e Hobson 2012):

$$\delta X\ (‰) = (R_{sample}/R_{standard}) - 1 \quad (1)$$

onde X é o valor de ^{13}C ou ^{15}N, e R corresponde a razão $^{13}C/^{12}C$ ou $^{15}N/^{14}N$ (Peterson e Fry 1987). Em ambos os laboratórios, são utilizados padrões internos de composição isotópica conhecida a fim de estimar a precisão do equipamento. A precisão analítica (desvio padrão - DP) dos padrões internos é de < 0,2‰ para $\delta^{15}N$ e < 0,04‰ para $\delta^{13}C$ em UNM-CSI; e de < 0,1‰ para $\delta^{13}C$ e $\delta^{15}N$ em SICL–WSU.

Amostras analisadas em laboratórios distintos podem ser comparadas diretamente após uma calibração. Valores isotópicos de penas de albatroz-do-bico-amarelo *Thalassarche chlororhynchos* revelaram diferenças significativas nos valores de $\delta^{13}C$ em amostras analisadas em ambos os laboratórios UNM–CSI e SICL–WSU (Leal 2018). Por esta razão, os valores de $\delta^{13}C$ das amostras analisadas em SICL–WSU no presente estudo foram corrigidas utilizando a seguinte equação:

$$\delta^{13}C_{corrigido} = (-1,59) + 0,92\ (\delta WSU) \quad (2)$$

onde δWSU representa os valores de δ^{13}C obtidos SICL–WSU (Leal 2018).

Análise de dados

Os valores isotópicos das amostras de pele com e sem extração de lipídios foram avaliados separadamente para os valores de δ^{13}C e δ^{15}N através do teste *t* pareado. Para verificar se há diferença significativa dos valores isotópicos entre as classes de tamanho (i.e., adultos e juvenis) e a composição genética (i.e., a classificação haplotípica), foi aplicado um modelo linear generalizado (GLM). Os machos foram considerados juvenis quando o CCC era menor que o tamanho mínimo estimado (88,2 cm CCC) para machos do Atlântico Norte que já atingiram a maturidade sexual (Avens et al. 2015). O GLM foi ajustado utilizando uma distribuição Gamma, em que os valores de δ^{13}C e δ^{15}N foram as variáveis resposta, e as classes de tamanho e a classificação haplotípica as variáveis explicativas categóricas com dois e cinco níveis, respectivamente. As variáveis que apresentaram diferenças significativas nos valores isotópicos foram utilizadas para separar os machos de tartarugas-cabeçudas em grupos distintos. O nível de significância dos testes estatísticos (teste *t* e GLM) foi de $\alpha = 0,05$.

Para cada grupo definido através do GLM, foi estimada a contribuição relativa das presas na dieta dos machos de tartaruga-cabeçuda através de modelos Bayesianos de mistura isotópica (Stable Isotope Mixing Models - SIMM) através do pacote "simmr" (Parnell 2016) desenvolvido para o software R. Antes da análise com o SIMM, três fatores de discriminação trófica (FDT) previamente descritos, bem como a viabilidade do banco de dados de presas, foram avaliados através de simulações de polígonos de mistura com base na inferência estatística Bayesiana, através dos pacotes "sp" e "splancs" (Smith et al. 2013). A simulação do polígono de mistura é visualizada através de uma elipse limitada pelos valores isotópicos das fontes alimentares, fornecendo uma base quantitativa para excluir consumidores (aqueles que ficam fora dos limites do polígono com Intervalo de Credibilidade - CrI de 95%) e a avaliação dos FDTs mais apropriados (Smith et al. 2013). Um dos FDTs avaliados é proveniente de um estudo conduzido com tartarugas-cabeçuda de cativeiro, o qual estimou os valores médios do tempo de residência e o FDT em vários tecidos (Reich et al. 2008). Para a pele, foi estimado um tempo de residência de 46,1 ± 8,9 dias para o δ^{13}C e 44,9 ± 3,1 dias para o δ^{15}N. O FDT foi estimado em 1,11 ± 0,17‰

para o δ^{13}C e 1,60 ± 0,07‰ para o δ^{15}N. Os outros dois FDTs utilizados nas simulações foram estimados a partir de valores isotópicos da pele de juvenis e adultos de tartaruga-verde criadas em cativeiro em Cayman Turtle Farm, Antilhas (Vander Zanden et al. 2012). Os valores estimados para os juvenis foi 1,87 ± 0,56‰ para o δ^{13}C e 4,77 ± 0,40‰ para o δ^{15}N, e para os adultos foram 1,62 ± 0,61‰ para δ^{13}C e 4,04 ± 0,04‰ para o δ^{15}N. Ao aplicar o SIMM, uma matriz de diagnóstico é gerada, informando a correlação entre as fontes alimentares. Esta matriz possibilita identificar se o modelo ajustou adequadamente ou se o modelo não diferenciou os itens alimentares (Parnell 2016). Com base nesta matriz, os crustáceos *L. spinosa* e *D. insignis*, e os peixes *M. furnieri* e *P. brasiliensis* foram agrupados de acordo com a similaridade dos valores isotópicos, levando em conta o habitat e o tipo de alimentação dos organismos (Phillips et al. 2005).

SÍNTESE DOS RESULTADOS

No Capítulo 1, a composição genética dos estoques mistos de tartaruga-cabeçuda foi avaliada através de sequências de 780 pb da região controle do mtDNA. As áreas de estudo exibiram indivíduos de diferentes fases de vida, mas os resultados indicaram que as áreas de forrageio oceânicas são habitadas por tartarugas significativamente menores do que as de habitats neríticos. Nos estoques mistos de tartaruga-cabeçuda do ASO, foram observados 13 haplótipos previamente descritos entre os 407 indivíduos amostrados (Fig. 4): CC-A1.1, CC-A2.1, CC-A3.1, CC-A4.1, CC-A4.2, CC-A4.3, CC-A10.1, CC-A11.6, CC-A17.1, CC-A24.1, CC-A25.1, CC-P1.1, CC-P5. Além disso, foram identificados cinco haplótipos órfãos, ou seja, haplótipos que não foram registrados previamente nas áreas de desova de tartaruga-cabeçuda já estudados. Um dos haplótipos consiste em uma variação do haplótipo CC-A11.6, e os outros quatro são variações do haplótipo CC-P5. Na região costeira do nordeste e sul do Brasil, também foram observados espécimes com haplótipos característicos de tartaruga-oliva, indicando hibridização, isto é, indivíduos descendentes do cruzamento de duas espécies distintas de tartarugas marinhas.

As análises de diferenciação genética mostraram que os estoques mistos amostrados na região costeira do sul do Brasil são geneticamente distintos daqueles da região costeira do nordeste e oceânica do sul do Brasil. Os resultados do MSA revelaram que todas as áreas de alimentação amostradas, tanto no ambiente costeiro como no oceânico, são compostas em maior proporção de tartarugas-cabeçuda provenientes de áreas de desova do Brasil. As contribuições das áreas de desova brasileiras foram

similares entre as áreas de alimentação neríticas, mas na região oceânica observou-se uma maior contribuição de indivíduos originários do estado do Espírito Santo. Além disso, na região nordeste foi observada em menor proporção a contribuição de colônias do México (Atlântico Norte) e Cabo Verde (leste do Atlântico), enquanto que os estoques mistos do sul do Brasil, principalmente os da região oceânica, são compostos por indivíduos provenientes de Cabo Verde, Omã (Oceano Índico) e Nova Caledônia (sul do Pacífico).

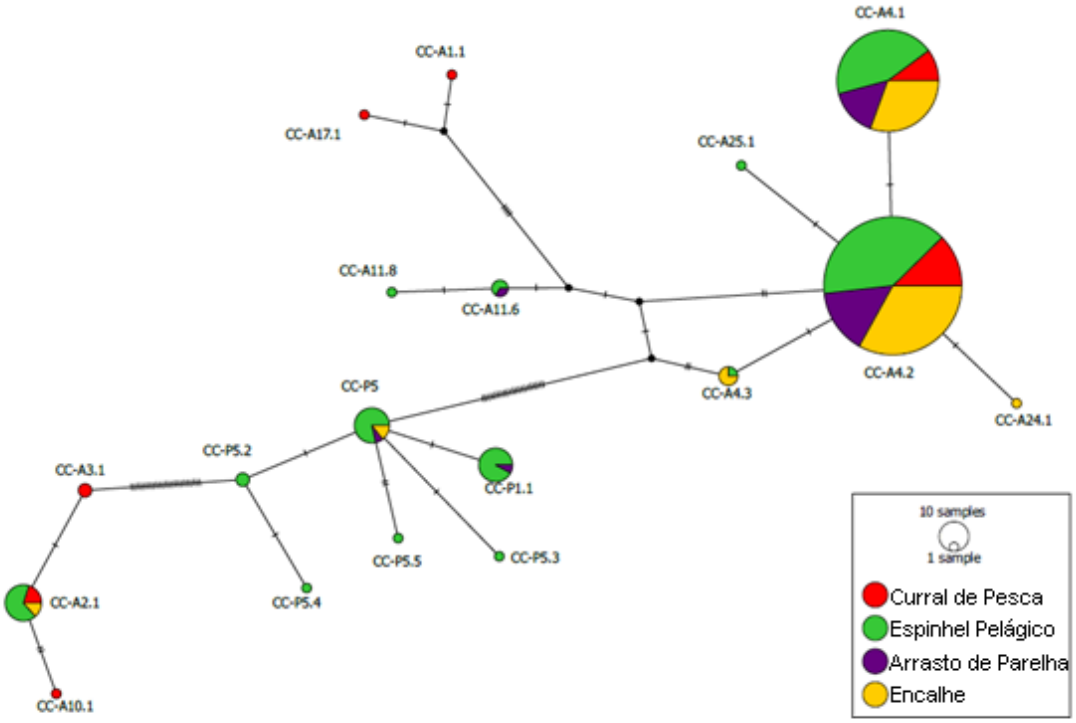

Fig. 4. Rede dos haplótipos de tartaruga-cabeçuda observados nas áreas de alimentação do Atlântico Sul Ocidental. O tamanho dos círculos representa as frequências haplotípicas e as cores indicam a fonte de amostragem.

No Capítulo 2, foram amostrados 26 machos (19 adultos e 7 juvenis) entre os espécimes de tartaruga-cabeçuda encalhados ao longo da região costeira do sul do Brasil. Sequências de 818 pb do mtDNA indicaram que 88,5% dos indivíduos amostrados eram provenientes de áreas de desova do Brasil, principalmente da região sudeste do país (Espírito Santo e Rio de Janeiro). Os resultados também revelaram pela primeira vez a presença de machos híbridos. Neste caso, o evento de hibridização observado ocorreu entre a tartaruga-cabeçuda e a tartaruga-oliva.

A análise de isótopos estáveis de carbono e nitrogênio da pele dos machos indicou diferenças na alimentação e uso do habitat entre machos adultos e juvenis. Os machos adultos utilizam preferencialmente a região costeira e alimentam-se em maior proporção de invertebrados bentônicos, como o ermitão *Loxopagurus loxochelis* e o gastrópode *Buccinanops monoliferum*. Já os juvenis apresentaram uma alta variabilidade nos valores isotópicos, revelando o uso do ambiente costeiro e do ambiente oceânico durante esta fase de vida. Para estes indivíduos, tanto as presas pelágicas (por exemplo, salpas), como as bentônicas (ermitão *L. loxochelis*) destacaram-se como os itens que mais contribuem na alimentação dos juvenis.

No Capítulo 3, a análise de 9 *loci* de microssatélites de tartarugas-cabeçuda das áreas de desova do ASO revelou uma alta diversidade genética entre as tartarugas amostradas, com uma alta proporção de alelos raros. Os valores de F_{is} foram negativos nos dois níveis populacionais avaliados, indicando um alto fluxo gênico, e baixa taxa de endocruzamento entre os indivíduos da população. Não foi observada diferenciação genética entre as colônias através dos índices de distância genética (F_{st} e D_{st}), nem na análise de isolamento por distância geográfica. No entanto, a análise Bayesiana de agrupamento identificou dois agrupamentos entre as colônias, em que Sergipe e Espírito Santo são geneticamente distintos de Bahia e Rio de Janeiro.

As análises de gargalo populacional baseadas na frequência de alelos raros na população revelaram uma deficiência na colônia de Sergipe, compatível com evento de gargalo recente. A estimativa de excesso de heterozigosidade não revelou perda significativa de diversidade genética e indicou que as colônias avaliadas estão em equilíbrio de mutação e deriva. Os valores da razão-*M*, no entanto, mostram sinal de gargalo nos dois níveis populacionais avaliados, sugerindo um desequilíbrio demográfico nas últimas gerações.

CONCLUSÕES

No presente estudo, foi realizada uma ampla análise genética de tartaruga-cabeçuda nas principais áreas reprodutivas no ASO e em áreas de alimentação no nordeste e sul do Brasil, locais onde operam distintas artes pesqueiras. Com base nos dados obtidos nesta tese, conclui-se que:

- As atividades pesqueiras que atuam no ASO estão impactando as populações de tartaruga-cabeçuda provenientes de todas as bacias oceânicas, mas principalmente as que compõem as populações do Brasil;

- O fato de uma alta proporção de tartarugas-cabeçudas originárias de colônias brasileiras ocuparem as águas jurisdicionais do Brasil em diferentes fases de vida e habitats pode representar uma vantagem do ponto de vista de manejo das atividades pesqueiras, facilitando a aplicação e fiscalização de ações que promovam a conservação da população do ASO;

- A contribuição estimada das colônias do Atlântico Norte e Leste e do Indo-Pacífico sugerem que as correntes oceânicas superficiais exercem influência na composição dos estoques mistos no ASO;

- Os machos de tartaruga-cabeçuda apresentam padrões distintos de uso de habitat e alimentação entre adultos e juvenis;

- A alta diversidade genética e a baixa estruturação observada entre as colônias brasileiras indicam um alto fluxo gênico entre as áreas, provavelmente mediada pelos machos;

- Embora as análises de gargalo populacional indiquem que, de maneira geral, a população está em equilíbrio, há indícios de que a população do ASO enfrentou uma drástica redução nas últimas gerações, a qual deve ser considerada na implementação e/ou continuidade de medidas de conservação.

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CAPÍTULO 1

Genetic structure and origins of loggerhead sea turtle foraging aggregations in the southwestern Atlantic Ocean: fisheries interactions and conservation implications

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Genetic structure and origins of loggerhead sea turtle foraging aggregations in the southwestern Atlantic Ocean: fisheries interactions and conservation implications

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Abstract

Fishery activities are known to be the highest threat to loggerhead sea turtles (*Caretta caretta*) in foraging grounds. In the southwestern Atlantic Ocean some fisheries frequently overlap with the main foraging grounds of loggerheads. Generally, feeding aggregations are composed by individuals from multiple nesting grounds. The present study assessed the genetic structure and natal origins of loggerhead turtles in foraging aggregations in northeastern and southern Brazil. Tissue samples were collected from turtles incidentally caught in coastal fish weirs (northeastern), in neritic bottom pair trawl fisheries, offshore in pelagic longline fisheries, and washed ashore (southern). Based on mitochondrial DNA control region sequences, moderate genetic structure was observed between aggregations in the Northeast and South, as well as between turtles from coastal and oceanic waters. A Bayesian mixed stock analysis showed that Brazilian rookeries are the main contributors to foraging aggregations in all sampled areas. In northeastern Brazil, haplotypes from the North and East Atlantic were also observed, whereas in southern Brazil, individuals from Indian and Pacific nesting grounds were identified, mainly among early juveniles in oceanic waters. Furthermore, hybrid individuals with olive ridley (*Lepidochelys olivacea*) were observed among turtles caught in fish weirs and washed ashore. These results reveal the importance of Brazilian foraging grounds to the

maintenance of genetic diversity and stability of loggerhead populations in Brazil and worldwide, and highlight that future fisheries management plans must consider time-closure areas in southern Brazil to reduce sea turtles bycatch in both neritic and oceanic habitats.

Keywords: Brazil; bycatch; foraging grounds; mitochondrial DNA.

1. Introduction

The loggerhead sea turtle (*Caretta caretta*) is widely distributed throughout tropical and subtropical regions of the Atlantic, Pacific and Indian Oceans, as well in the Mediterranean Sea (Casale and Tucker, 2017). The life history of this species encompasses migrations at large spatial scales and the use of distinct habitats (neritic *vs*. oceanic) throughout their ontogenetic development (Bolten, 2003; Snover et al., 2010) and between reproductive seasons (Plotkin, 2003). Generally, juveniles spend their early life stage in the open sea, which is followed by recruitment to neritic habitats where turtles remain during late juvenile and adult stages (Bolten, 2003). Despite this general pattern, variation in habitat use and migrations occurs in response to changes in sea surface temperature and availability of food resources (Mansfield et al., 2009; McClellan et al., 2010; Pajuelo et al., 2016; Saito et al., 2015). Throughout its life cycle, this species is exposed to different anthropogenic threats (Clusa et al., 2016; Lutcavage et al., 1997; Monteiro et al., 2016; Rizzi et al., 2019), being currently classified as *Vulnerable* on the IUCN Red List (Casale and Tucker 2017). Therefore, understanding the patterns of habitat use and spatial connectivity is crucial for the conservation of the species and for the prevention and mitigation of threats.

One of the main threats to loggerhead sea turtles in the ocean is bycatch in fisheries (Wallace et al., 2010). Bycatch is defined as the incidental capture of non-target species and its occurrence has been reported in longline, driftnets, gillnets, trawls and fishing weirs (Casale et al., 2017; Clusa et al., 2016; Fiedler et al., 2012; Lima et al., 2013; Monteiro et al., 2016; Sales et al., 2008). In the southwestern Atlantic Ocean (SWA), for example, it was estimated that 6594 loggerheads were incidentally caught in pelagic longline fisheries in Brazilian and adjacent Uruguayan waters, from 1998 to 2010 (Giffoni et al., 2014). The SWA holds numerous fishing activities that overlap with the distribution of loggerhead foraging grounds (Monteiro, 2017). Satellite tracking data indicated that

after the breeding season, adult loggerhead females from nesting grounds in Bahia state, northeastern Brazil, migrate further northwards along the coast to foraging grounds, mainly at Ceará state (CE), and display high fidelity to these areas (Marcovaldi et al., 2010). A long-term fishery monitoring study also reported the occurrence of large juveniles feeding in the neritic zone of CE, where artisanal weir fisheries are common (Lima et al., 2013). In southern Brazil, previous studies have revealed that large loggerhead juveniles and adults inhabit coastal waters (Medeiros et al., 2019; Monteiro et al., 2016), whereas early juveniles use oceanic habitats for feeding and development (Barros, 2010; Petitet et al., 2012). At this region, both neritic and oceanic industrial fisheries (e.g. bottom pair trawl and pelagic longline, respectively) incidentally capture sea turtles (Monteiro, 2017; Sales et al., 2008), resulting in thousands of dead loggerhead stranding in southern Brazil over the last two decades (Monteiro et al., 2016).

Foraging aggregations of sea turtles are usually mixed stocks, i.e., composed by individuals originating from distinct nesting populations worldwide (Clusa et al., 2016, Rees et al., 2017; Tolve et al., 2018; but see Prosdocimi et al., 2015). These origins can be assessed through the analysis of mitochondrial DNA (mtDNA), a maternally inherited marker, since sea turtle populations are significantly structured in terms of maternal lineages due to the high fidelity of adult females to their natal areas (natal philopatry) (Jensen et al., 2013; Shamblin et al., 2014). Studies based on mtDNA have revealed that loggerheads occurring in neritic habitats of Uruguay and Argentina originate exclusively from Brazilian rookeries (Caraccio et al., 2008; Prosdocimi et al., 2015), whereas foraging aggregations in oceanic waters off Uruguay also hold a low proportion of individuals coming from North Atlantic, Mediterranean, and Pacific nesting grounds (Caraccio et al., 2008). Similar natal origins were observed in loggerheads caught in oceanic pelagic longline off southern Brazil (Reis et al., 2010), but also including individuals from the Indian Ocean (Shamblin et al., 2014). Nevertheless, there is insufficient information on origins and structure of loggerhead foraging grounds in Brazil, especially those that overlap with fisheries in neritic habitats. In the present study, mtDNA sequences were analyzed from loggerhead sea turtles interacting with fisheries in northeastern and southern Brazil in order to estimate natal origins and genetic structure of foraging grounds in neritic and oceanic habitats.

2. Methods

2.1. Sample collection

Samples of skin and/or muscle were taken from loggerhead sea turtles caught in coastal fish weirs ($N = 53$) and pair bottom trawl ($N = 54$), and offshore in pelagic longline fisheries ($N = 181$), representing foraging aggregations from northeastern and southern Brazil (Fig. 1). Sample collection occurred from May 2005 to May 2016 in fish weir, December 2013 to April 2017 in pair bottom trawl, and November 2005 to June 2017 in pelagic longline. Loggerheads washed ashore were also sampled ($N = 119$) at the southern coast of Rio Grande do Sul state, between Lagoa do Peixe (31°21'S; 051°05 'W) and Arroio Chuí (33°44' S, 53°22' W), from October 2013 to December 2017 (Fig. 1). Although some stranded loggerheads showed evidence of fishing-related mortality, the cause of death of most specimens was not possible to determine in the field. Therefore, beach monitoring was assumed as a distinct source of sampling. For each specimen, curved carapace length (CCL) was measured with a flexible metric tape (± 0.1 cm), from the mid-point of the nuchal scute to the end of the posterior marginal scute (Bolten, 1999). Tissue samples were collected using a sterile scalpel or 6-mm biopsy punch, stored in sodium chloride (NaCl), absolute ethanol or DMSO solution, and frozen at -20°C until laboratory procedures. Samples were collected as part of beach and fisheries monitoring programs and different collection and storage protocols were applied according to partner institutions that conducted the sampling: Núcleo de Educação e Monitoramento Ambiental - NEMA, and Fundação Pró-TAMAR.

2.2. *Laboratory procedures*

Genomic DNA was extracted with PureLinkTM Genomic DNA Kits. A 780 base-pair (bp) fragment of the mtDNA D-loop region was amplified through Polymerase Chain Reactions (PCR) with the primer pair LCM15382 (5 - GCT TAA CCC TAA AGC ATT GG - 3') and H950 (5 - GTC TCG GAT TTA GGG GTT TG - 3′) (Abreu-Grobois et al., 2006). The final reaction volume was 50µl, containing 20 to 50 ng of genomic DNA, 5U of Platinum® Taq Polymerase (Invitrogen) or Recombinant Taq Polymerase, 0.2 µM of each primer, 0.4 mM of dNTPs, 1x PCR Buffer and 0.1 mM $MgCl_2$. PCRs were performed in an Applied Biosystems Veriti 96-well Thermocycler under the following conditions: denaturation of 5 min at 94ºC; 36 cycles of 30 s at 94ºC, 30 s at 50ºC, 1 min

at 72ºC; and a final extension of 10 min at 72ºC. Negative controls were included in PCRs to detect contaminations during the amplification process. The obtained PCR products were purified with PureLink^{TM} Quick Gel Extraction & Purification Combo Kits. Purified PCR products were sequenced in forward and reverse directions using a ABI PRISM 3730XL Analyzer.

2.3. Data analysis

Variations in biometric measurements among sampled loggerheads were evaluated using a Kruskal-Wallis non-parametric test, followed by Wilcoxon rank sum test pairwise comparisons to verify significant differences, in R 3.4.2 (R Core Team, 2017). The obtained mtDNA sequences were edited using BioEdit 7.0.9 (Hall, 1999), and haplotypes were classified by comparing to previously described haplotypes recorded in the Archie Carr Center for Sea Turtle Research (ACCSTR) (http://accstr.ufl.edu/) and GenBank (http://ncbi.nlm.nih.gov) databases. Throughout this paper, haplotypes are referred according to standardized nomenclature established in both databases. Haplotype (h) and nucleotide diversity (π) (Nei, 1987) were calculated for each sampling location using DNAsp 5.10 (Rozas et al., 2003). Genetic studies analyzing longer mtDNA fragments (~800pb) are recent and there is little information on standard diversity indices based on long sequences for loggerheads (Prosdocimi et al., 2015; Shamblin et al., 2014). Therefore, diversity indices were calculated for both short (360pb) and long sequences (780pb) to compare with previous studies carried out for loggerheads from foraging grounds in the SWA. Genetic differentiation among sampling areas was verified in ARLEQUIN v. 3.1 (Excoffier et al., 2005) based on pairwise fixation indices F_{st} and ϕ_{st}, using only haplotype frequencies and the Tamura-Nei model of nucleotide substitution, respectively. The most adequate model of nucleotide substitution to use in ϕ_{st} estimates was defined through jModelTest 2.1.10 (Darriba et al., 2012). The degree of genetic differentiation based on F_{st} and ϕ_{st} values were considered low, moderate, high and very high when ranging from 0-0.05, 0.05-0.15, 0.15-0.25, and >0.25, respectively (Wright, 1978). A median joining network was constructed using PopArt v1.7 (Bandelt et al., 1999) to illustrate the frequency and the relationship among haplotypes of sampled areas.

A 'many-to-many' Bayesian Mixed Stock Analysis (MSA) was performed in order to estimate the contributions of loggerhead rookeries worldwide to the SWA foraging grounds (Pella and Masuda, 2001). MSA was carried out in R through the

mixstock package (Bolker et al., 2007). The haplotype frequencies reported in management units of loggerheads in the Mediterranean Sea, Atlantic, Pacific and western Indian Oceans were used as baseline (Fig. 2; Table S1 and S2; Boyle et al., 2009; Matsuzawa et al., 2016; Shamblin et al., 2014). The baseline matrix was also complemented with haplotype frequency data of nesting females ($N = 81$) and hatchlings ($N = 21$) from Brazilian rookeries (unpublished data). Orphan haplotypes and hybrid individuals, which were not present in baseline data from loggerhead rookeries, were excluded from MSA (Bolker et al., 2007). A Markov Chain Monte Carlo (MCMC) was applied to obtain posterior distributions, integrating the data likelihood with an uninformative prior, through four chains of 120,000 interactions with an initial burn-in of the first 20,000, resulting in posterior distributions with 95% credibility intervals. The variance between chains was assessed by the Gelman-Rubin reduction factor, in which values below 1.2 for all parameters indicate that convergence in the algorithm was achieved and the posterior distributions were reliable (Bolker et al., 2007). The MSA satisfied the Gelman–Rubin criteria, resulting in a shrink factor of 1.1.

3. Results

The mean CCL of specimens caught in weir, pair trawl and pelagic longline fisheries was 73.8 ± 6.9 cm (range 57.7-98.5 cm), 75.6 ± 10.6 cm (53.0-99.5 cm), and 67.4 ± 9.4 cm (37.5-98.0 cm), respectively. The mean CCL of stranded loggerheads was 78.9 ± 19.0 cm (11.0-114.0 cm). The Kruskal-Wallis test showed that loggerheads incidentally caught in oceanic feeding aggregation was significantly smaller than those from neritic areas ($H_3 = 117.99$; $P < 0.01$; Fig. 3).

Thirteen variants of previously reported haplotypes were observed among 407 loggerhead turtles sampled (Table 1). Additionally, five orphan haplotypes (i.e., haplotypes that were not previously reported in loggerhead rookeries) were recorded: one haplotype is a variant of CC-A11.6, and the other four are variants of CC-P5 (Table 1; Fig. 4). Furthermore, mtDNA haplotypes characteristic of olive ridley (*Lepidochelys olivacea*) were observed, hereafter referred to as Cc × Lo haplotypes, indicating hybridization between species (Table 1).

Standard diversity indices are summarized in Table 2. Cc × Lo haplotypes were not included in diversity estimates, due to the high proportion of polymorphic sites that

would overestimate genetic diversity (Reis et al., 2010). F_{st} values indicated low genetic differentiation between stranded loggerheads and individuals from pelagic longline bycatch in South Brazil, but did not reveal difference among other sampled areas (Table 3). ϕ_{st} values indicated moderate significant genetic divergence between turtles caught in coastal fisheries in the Northeast (fish weir) and in the South (bottom pair trawl), as well as between loggerheads from neritic and oceanic habitats in southern Brazilian (Table 3). No significant difference was observed between turtles sampled in the bottom pair trawl fishery and washed ashore.

Posterior distributions of MSA indicated that Brazilian rookeries contribute in similar proportions to both northeastern and southern foraging grounds (Table S3; Fig. 5). In the Northeast, loggerhead turtles also originated in lower proportions from nesting grounds at Cape Verde islands (East Atlantic) and Mexico Gulf (North Atlantic) (Fig. 5a). In the South, natal origin estimates of specimens revealed that, despite the dominance of Brazilian origins, rookeries from Cape Verde islands, New Caledonia (southern Pacific) and Oman (Indian Ocean) also contributed to oceanic (Fig. 5b) and neritic foraging grounds in southern Brazil (Fig. 5c and d).

4. Discussion

Loggerhead sea turtles are highly migratory throughout their life cycles and often occupy several habitats under the jurisdiction of multiple countries. In this context, genetic analyses stand out as an important tool for understanding connectivity, structure and migrations of sea turtle populations, and to ultimate promote conservation strategies. The present study provides a broad genetic analysis of loggerhead sea turtles that interact with fisheries in SWA, assessing previously undescribed feeding aggregations in neritic habitats, providing complementary knowledge on oceanic feeding areas through long mtDNA sequences, and identifying the contributions of multiple loggerhead rookeries to mixed stocks in feeding areas of northeastern and southern Brazil. The study areas exhibited individuals of different life stages, but results indicated that oceanic foraging grounds are inhabited by turtles significantly smaller than turtles from neritic habitats. This shows that fishing activities can affect loggerhead sea turtles throughout their entire life cycle, potentially disturbing population stability and viability (Heppel et al., 1998).

A heterogeneous genetic composition of population stocks was observed within SWA, with foraging aggregations showing distinct genetic profiles between northeastern/southern, and neritic/oceanic habitats. Fisheries operating in the SWA are impacting loggerhead populations from all ocean basins, but mainly those from Brazilian nesting grounds. All studied foraging aggregations showed high frequency of endemic haplotypes from Brazilian rookeries (Fig. 4; Table S2), which exhibit unique genetic profiles, showing haplotypes that are not reported in other rookeries around the world (Reis et al., 2010; Shamblin et al., 2014). The high residence of Brazilian individuals in developmental and feeding grounds in the SWA can represent an advantage for the population by minimizing energy expenditure with long migrations that can be allocated directly to somatic growth and reproduction. Furthermore, the fact that loggerhead turtles inhabit Brazil's jurisdictional waters throughout their entire life cycle stands out as an advantage from a management perspective, facilitating the application and enforcement of conservation actions for Brazilian populations.

Nevertheless, the mixed stocks also host loggerheads from distant natal origins. In northeastern Brazil, loggerheads caught in fish weirs showed haplotypes from North and eastern Atlantic nesting grounds such as the rookeries on the southeastern coast of the United States and the southern Gulf of Mexico (CC-A1.1 and CC-A10.1), and the rookeries at Cape Verde (CC-A17.1). Individuals also presented the haplotype CC-A3.1, which is shared between North Atlantic and Mediterranean Sea rookeries (Table S2; Shamblin et al., 2014). On the other hand, in southern Brazil haplotypes from rookeries located in the Pacific (CC-P1.1 and CC-P5) and Indian (CC-A11.6) Oceans, were observed mainly in offshore feeding grounds. Haplotypes CC-P1.1 and CC-P5 characterize Pacific loggerhead populations nesting in western Australia and southern Japan (Boyle et al., 2009; Matsuzawa et al., 2016). CC-A11.6 has been recognized as a variant of Atlantic lineage that colonized the Indian Ocean, mainly at Oman nesting sites (Shamblin et al., 2014). The presence of new variants of Indian and Pacific haplotypes indicates that these ocean basins require further studies to increase genetic characterization of rookeries and to ensure adequate population assessment. Haplotype CC-A2.1, observed among loggerheads from both northeastern and southern Brazil, is the most geographically widespread loggerhead haplotype present in all northwestern/eastern Atlantic and Mediterranean rookeries, as well as in South Africa (Garofalo et al., 2009; Monzón-Argüello et al., 2010; Shamblin et al., 2014).

Genetic analysis has been previously used to estimate genetic diversity and identify the natal origins of loggerheads in the SWA. Genetic diversity indices based on short mtDNA sequences showed higher values in mixed stocks from coastal waters off Brazil (Table 2) than off Argentina (h = 0.032 ± 0.031 and π = 0.000089 ± 0.0003; Prosdocimi et al., 2015). Moreover, genetic indices previously reported for loggerheads caught in pelagic longline in southern Brazil were higher (h = 0.714 ± 0.031 and π = 0.017 ± 0.001; Reis et al., 2010) than values found in the present study. Mixed stocks in oceanic waters off Uruguay and Brazil are composed by early juveniles from southwestern and North Atlantic, Mediterranean Sea, and Pacific Ocean (Caraccio et al., 2008; Reis et al., 2010), and the present work corroborate distant origins of offshore mixed stocks in the SWA. In coastal waters off Uruguay and Argentina, adults and large juveniles only reported haplotypes from Brazilian rookeries (Caraccio et al., 2008; Prosdocimi et al., 2015); however, the present study showed that neritic areas in Brazil can also be composed by individuals from distant origins, though in low frequencies.

Several factors are suggested to influence the movements of sea turtles, among which ocean currents are known to play an important part in the passive dispersal of hatchlings and early juveniles (Cardona and Hays, 2018; Mansfield and Putman, 2013), although directional migration also occurs according to environmental conditions, mainly among large juveniles and adults (e.g., sea surface temperature; Monteiro, 2017; Saito et al., 2015). MSA results suggest that major surface currents could influence the composition of loggerhead foraging grounds in the SWA, given the geographic location of the nesting grounds that contribute most to the studied mixed stocks. Previous genetic studies have suggested that loggerhead sea turtles colonized the SWA following a north to south route facilitated by the Brazil Current (Reis et al., 2010), and that the widespread distribution of Indian and Pacific Ocean haplotypes in the region is mediated by the Agulhas Current, which could aid the dispersal of juveniles to South America (Shamblin et al., 2014). In northeastern Brazil, distinct movements patterns were observed in early juvenile loggerhead turtles, influenced by seasonal latitudinal changes in the bifurcation of the South Equatorial Current into the Brazil and North Brazil Currents (Mansfield et al., 2017). Therefore, this current system, along with the Equatorial Counter Current, could influence the recruitment of juveniles from the North and East Atlantic to coastal foraging grounds in Brazil, carrying individuals from the Gulf of Mexico and Cape Verde. Furthermore, a study performed in southern Brazil tracked loggerhead juveniles with

satellite transmitters showed that most individuals remained close to shore during spring and summer, when the tropical waters of the Brazil Current predominate, while some individuals moved northward or offshore during the austral autumn/winter to avoid the intrusion of cold waters carried by the Malvinas/Falkland Current (Monteiro, 2017).

Although the MSA provided valuable insights on the movements and origin of loggerheads sea turtles, this analysis has some limitations associated to the haplotype composition of the source populations, and results must be interpreted with caution. The occurrence of haplotypes that are widespread among rookeries, but present in low frequencies in feeding grounds, lead to large credibility intervals of contribution estimates (Jensen et al., 2013). Furthermore, according to haplotype frequencies in southern Brazil, the contribution of Cape Verde rookeries to the region seems to be overestimated and could indicate that the variance between chains are higher than within chains, although the Gelman-Rubin factor indicated convergence of model simulations (Gelman and Rubin, 1992). Additionally, the presence of orphan haplotypes at feeding areas indicates inadequate characterization of rookeries. To overcome these limitations, further analysis of loggerhead neritic foraging aggregations and rookeries, including increasing sample sizes for some sites, must be considered to improve baseline data and posterior distributions and better assignment of the management units that are under threat by fisheries.

The present work also reports Cc × Lo hybrids captured in fish weirs in the northeastern region and stranded along the southern coast of Brazil. Hybridization events between sea turtle species have been shown to occur in high frequencies at Brazilian nesting grounds (Lara-Ruiz et al., 2006; Reis et al., 2010), and the occurrence of Cc × Lo, as well as Cc × Ei (*Eretmochelys imbricata*) individuals, has also been observed in loggerhead foraging grounds in southern Brazil (Medeiros et al., 2019; Proietti et al., 2014). Although the observed olive ridley haplotypes are common at rookeries in Brazil and Guinea Bissau (haplotype F), and Suriname (haplotype E and F), Cc × Lo hybrids have only been reported at the Sergipe rookery (Reis et al., 2010). Considering this, and the high frequency of endemic Brazilian haplotypes observed in foraging aggregations, it is likely that the hybrid individuals caught in fish weirs and washed ashore originated from the Sergipe rookery.

The impact of sea turtle bycatch on management units is linked to the spatio-temporal overlap of fisheries with foraging grounds and the mortality caused by each type

of fishing gear, and considering distinct scenarios over wide geographical scales is crucial for the establishment of efficient management strategies (Clusa et al., 2016). In northeastern Brazil, it is reported that 99.6% of turtles caught in fish weirs are found alive (Lima et al., 2013), but some individuals are eventually killed by fishers for consumption (Silva et al., 2016). In southern Brazil, however, industrial fisheries severely impact sea turtles (Fiedler et al., 2012; Monteiro et al., 2016). Trawl fisheries overlap in about 75% with loggerhead feeding areas during the austral summer (Monteiro, 2017), and a 48% mortality rate due to bycatch was preliminarily estimated for this region (Monteiro et al., 2013), although this mortality is likely higher due to decompression sickness after the turtles have been released (García-Párraga et al., 2014; Parga et al., in prep.). High loggerhead bycatch rates have been also reported in pelagic longline fisheries in southern Brazil (Giffoni et al., 2014). Although the direct mortality rate associated with this fishery at the region is around 4% (Sales et al., 2008), the post-release mortality remains unknown but is likely higher, since the post-release mortality in longlines at the North Pacific Ocean has been estimated to be around 28% (Swimmer et al., 2013). Since the SWA holds high bycatch rates and high levels of fishing effort (Wallace et al., 2013), specific information on both instantaneous and post-release mortality rates of loggerheads in this region is required to assess demographic effects on populations worldwide.

The present study highlights that loggerhead stocks from the Atlantic, Indian and Pacific Oceans are being threatened by both oceanic and neritic fisheries, putting populations at risk since population reductions can result in losses of genetic diversity and evolutionary potential (Frankham et al., 1999). This seems particularly worrying in southern Brazil, where loggerhead sea turtle bycatch in industrial fishing fleets is high and constitutes the major cause of mortality and strandings of sea turtles (Monteiro et al., 2016). It is therefore crucial to implement bycatch mitigation strategies such as fishery time-area closures, along the continental shelf to reduce the impact of bottom trawl fisheries on large juvenile and adult males, and in offshore waters to minimize the bycatch of early juveniles in pelagic longlines. The use of circle hooks in longlines is also an effective mitigation for sea turtle bycatch: the implementation of this type of hook was associated with a 55% reduction of loggerhead turtles capture in the SWA (Sales et al., 2010). The application and enforcement of these conservation actions could reduce the impact of fisheries on loggerhead sea turtles, contributing to demography stability and genetic diversity increase, and promoting the conservation of loggerhead populations.

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Table 1 mtDNA haplotype frequencies of loggerhead sea turtle foraging grounds in Brazil. Frequencies represent turtles caught in fish weirs (northeastern Brazil), pelagic longline and bottom pair trawl fisheries (southern Brazil), and stranded along the southern coast of Brazil. GenBank database accession numbers of haplotypes are shown.

Haplotypes	Fish weir	Pelagic longline	Pair bottom trawl	Stranding	GenBank
CC-A1.1[a]	1				EU179436
CC-A2.1[a]	3	10		2	EU179445
CC-A3.1[b]	2				EU179455
CC-A4.1[c]	12	52	18	36	KF840723
CC-A4.2[c]	27	86	33	72	KF840725
CC-A4.3[c]		1		3	KF840724
CC-A10.1[b]	1				EU179440
CC-A11.6[c]		2	1		KF770994
CC-A11.8[*]		1			
CC-A17.1[a]	1				EU483082
CC-A24.1[c]				1	KF840726
CC-A25.1[d]		1			AY508990
CC-P1.1[e]		12	1		AB830477
CC-P5[f]		11	1	2	EF033113
CC-P5.2[*]		2			
CC-P5.3[*]		1			
CC-P5.4[*]		1			
CC-P5.5[*]		1			
Cc × Lo[g]	6			3	AF051773
Total	53	181	54	119	

[a] Monzón-Argüello et al., 2010; [b] Shamblin et al., 2012; [c] Shamblin et al., 2014; [d] Reis et al., 2010; [e] Matsuzawa et al., 2016; [f] Boyle et al., 2009; [g] Bowen et al., 1998. [*] Indicates orphan haplotypes.

Table 2 Standard diversity indices (mean ± SD) estimated for short and long mtDNA sequences of loggerhead sea turtles caught in fish weir, pelagic longline, pair bottom trawl, and washed ashore. *N* = number of individuals, *H* = number of haplotypes, *h* = haplotype diversity, π = nucleotide diversity.

Sequence size	Fish weir	Pelagic longline	Pair bottom trawl	Stranding
380pb				
N	53	181	54	119
H	6	10	4	4
h	0.3108 ± 0.0865	0.3893 ± 0.0453	0.1090 ± 0.0578	0.0851 ± 0.0358
π	0.0104 ± 0.0059	0.0113 ± 0.0062	0.0020 ± 0.0016	0.0023 ± 0.0018
780pb				
N	53	181	54	119
H	7	13	5	6
h	0.6105 ± 0.0628	0.6793 ± 0.0258	0.5241 ± 0.0481	0.5185 ± 0.0365
π	0.0104 ± 0.0054	0.0128 ± 0.0065	0.0031 ± 0.0019	0.0030 ± 0.0018

Table 3 Pairwise F_{st} (above diagonal) and ϕ_{st} (below diagonal) values of loggerhead sea turtles caught in fish weir, pelagic longline, pair bottom trawl, and stranded. Bold indicates significant difference ($P < 0.05$).

	Fish weir	Pelagic longline	Pair bottom trawl	Stranding
Fish weir	*	0.0271	**0.05607**	**0.06407**
Pelagic longline	0.00306	*	**0.05955**	**0.08016**
Pair bottom trawl	-0.00602	0.00877	*	-0.00829
Strandings	-0.0045	**0.01715**	-0.01103	*

Figure captions

Fig. 1. Sample sites in the southwestern Atlantic Ocean. Zoomed squares indicates the study area in northeastern (blue square) and southern (red square) Brazil. Abbreviations: CE = Ceará state; RS = Rio Grande do Sul state.

Fig. 2. Approximate location of loggerhead sea turtle nesting grounds in the (a) North Atlantic, (b) East Atlantic, (c) Mediterranean Sea, (d) North Pacific, and (e) South Pacific. Abbreviations: NUSA = South Carolina and Georgia coast, United States; CEFL = northeastern Florida; SEFL = southeastern Florida; DRSL = south Florida; QRMX = Quintana Roo, Mexico; KEY = Keewaydin Island, Florida; CSK = Casey Key, Florida; NWFL = northwestern Florida; SER = Sergipe, Brazil; BAH = Bahia, Brazil, ESP = Espírito Santo, Brazil; RIO = Rio de Janeiro, Brazil; BOA = Boa Vista, Cape Verde; SAL = Sal, Cape Verde; SLZ = Santa Luzia, Cape Verde; CAL = Calabria, Italy; WGRC = Greece; CRT = Crete; DYDL = Turkey; TKW = western Turkey; EMED = Cyprus, Lebanon and Israel; LIBY = Libya; NAT = South Africa; MAS = Oman; MAI = Mainland Japan; YAK = Yakushima, Japan; RYU = Ryukyu Archipelago, Japan; AUS = eastern Australia; NCL = New Caledonia. See details in Table S1.

Fig. 3. Curved carapace length (CCL) of loggerhead sea turtles incidentally captured in fisheries and stranded along the southern coast of Brazil. Boxes show interquartile range, solid black line show median value, and vertical lines are the maximum and minimum values

Fig. 4. Network for loggerhead sea turtle haplotypes recorded in the southwestern Atlantic Ocean. Circle sizes represent haplotype frequencies.

Fig. 5. Contribution proportion and 95% credibility interval of 30 stocks to foraging aggregations sampled in (a) fish weir, (b) pelagic longline, (c) bottom pair trawl, and from (d) stranding. Abbreviations: NUSA = South Carolina and Georgia coast, United States; CEFL = northeastern Florida; SEFL = southeastern Florida; DRSL = south Florida; QRMX = Quintana Roo, Mexico; KEY = Keewaydin Island, Florida; CSK = Casey Key, Florida; NWFL = northwestern Florida; SER = Sergipe, Brazil; BAH = Bahia, Brazil, ESP = Espírito Santo, Brazil; RIO = Rio de Janeiro, Brazil; BOA = Boa Vista, Cape

Verde; SAL= Sal, Cape Verde; SLZ = Santa Luzia, Cape Verde; CAL = Calabria, Italy; WGRC = Greece; CRT = Crete; DYDL = Turkey; TKW = western Turkey; EMED = Cyprus, Lebanon and Israel; LIBY = Libya; NAT = South Africa; MAS = Oman; MAI = Mainland Japan; YAK = Yakushima, Japan; RYU = Ryukyu Archipelago, Japan; AUS = eastern Australia; NCL = New Caledonia.

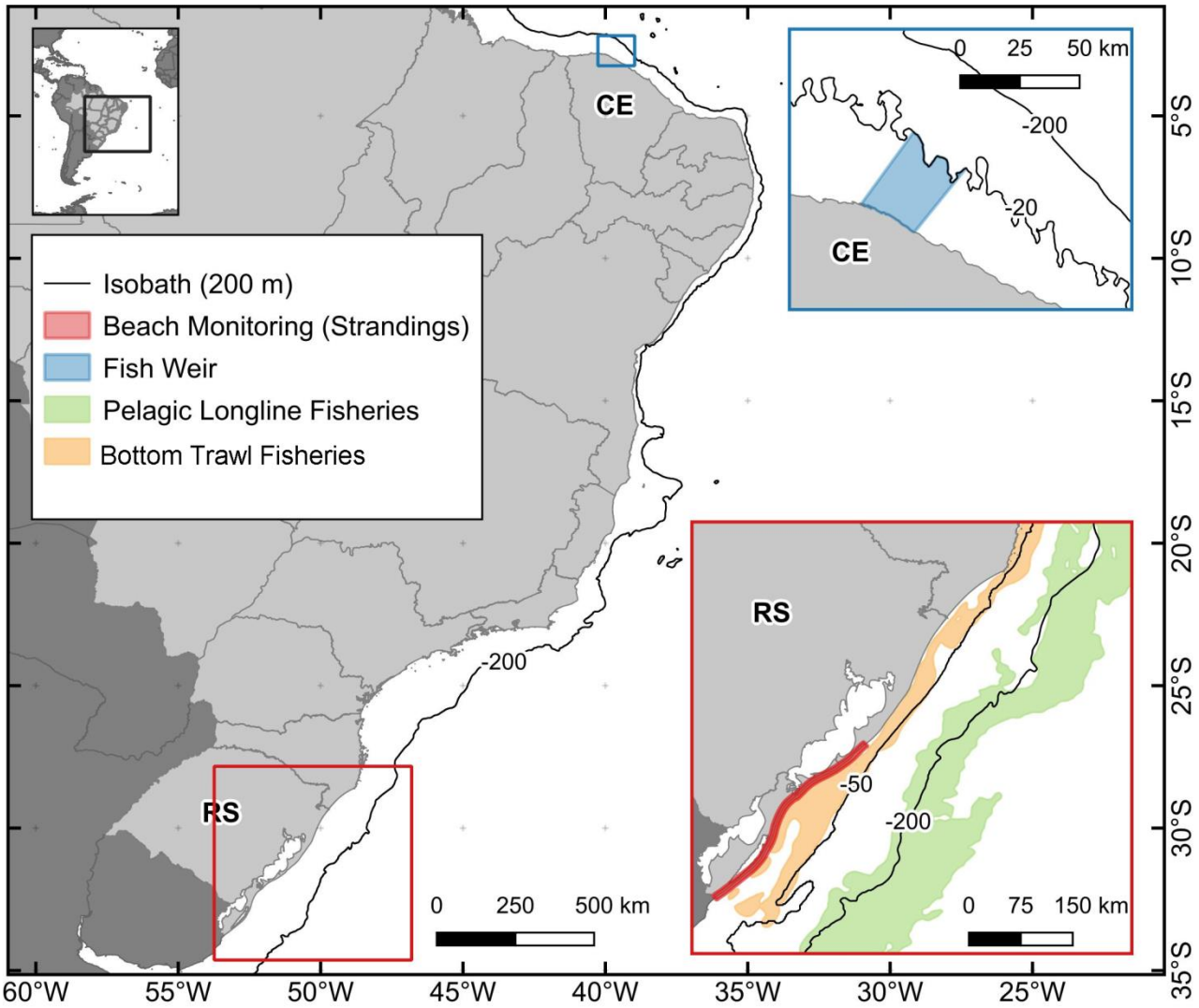

Isobath (200 m)
Beach Monitoring (Strandings)
Fish Weir
Pelagic Longline Fisheries
Bottom Trawl Fisheries
CE
RS
-200
-20
-50
0 25 50 km
0 250 500 km
0 75 150 km
5°S
10°S
15°S
20°S
25°S
30°S
35°S
60°W
55°W
50°W
45°W
40°W
35°W
30°W
25°W

Fig. 1.

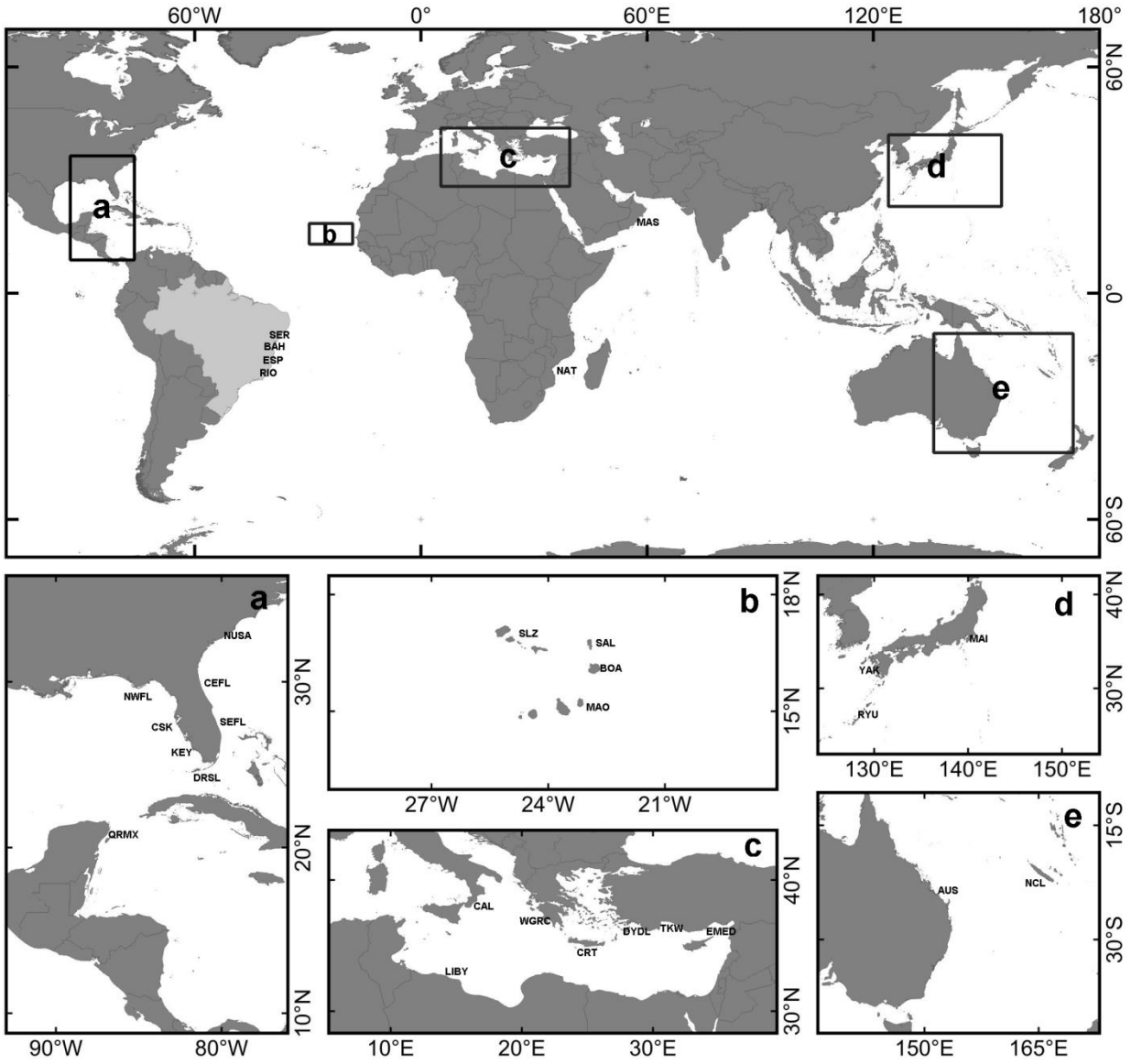

60°W
0°
60°E
120°E
180°
60°N
0°
60°S
a
b
c
d
e
SER
BAH
ESP
RIO
NAT
MAS
NUSA
CEFL
NWFL
SEFL
CSK
KEY
DRSL
QRMX
30°N
20°N
10°N
90°W
80°W
SLZ
SAL
BOA
MAO
18°N
15°N
27°W
24°W
21°W
CAL
WGRC
BYDL
TKW
EMED
CRT
LIBY
40°N
30°N
10°E
20°E
30°E
MAI
YAK
RYU
130°E
140°E
150°E
AUS
NCL
15°S
30°S
150°E
165°E

Fig. 2.

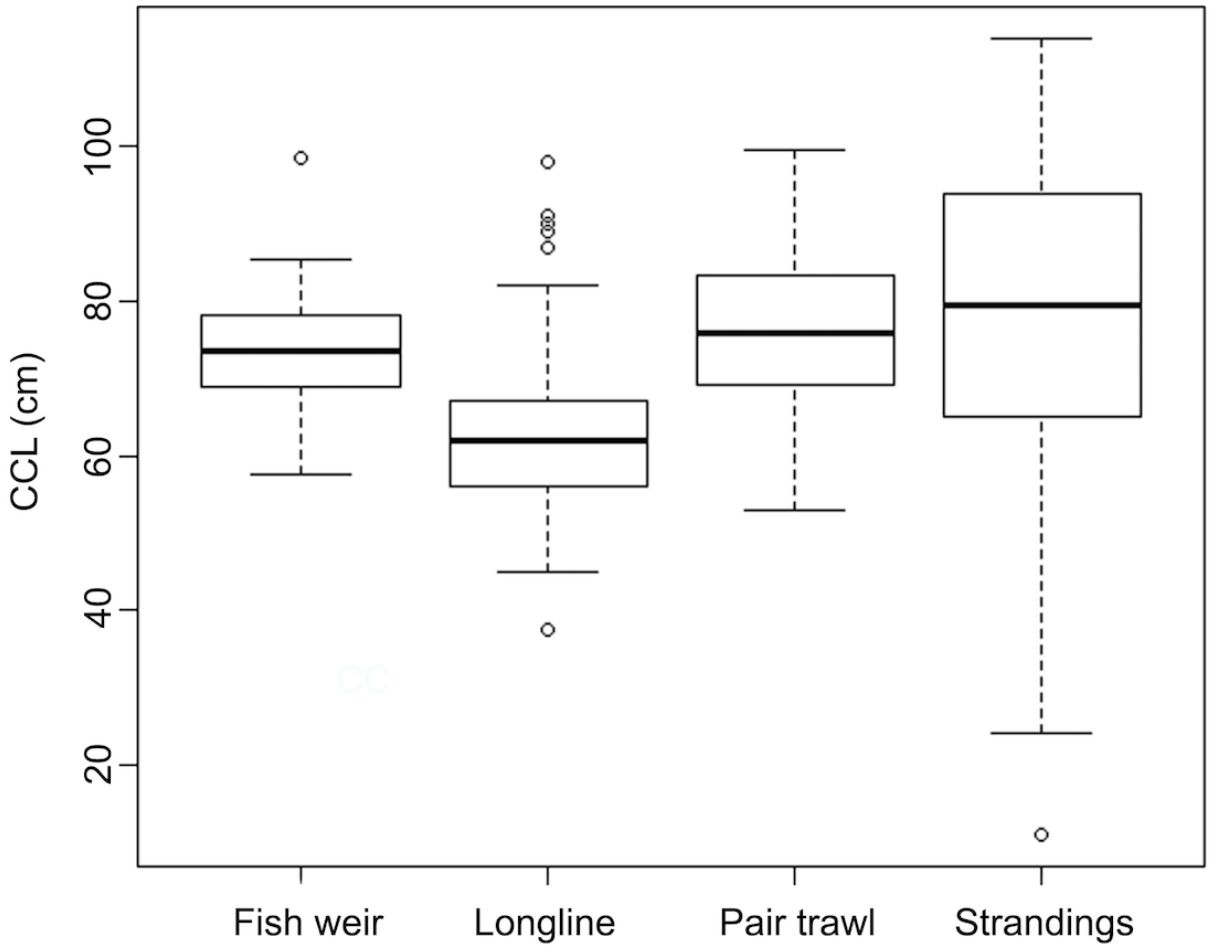

CCL (cm)
20
40
60
80
100
Fish weir
Longline
Pair trawl
Strandings

Fig. 3.

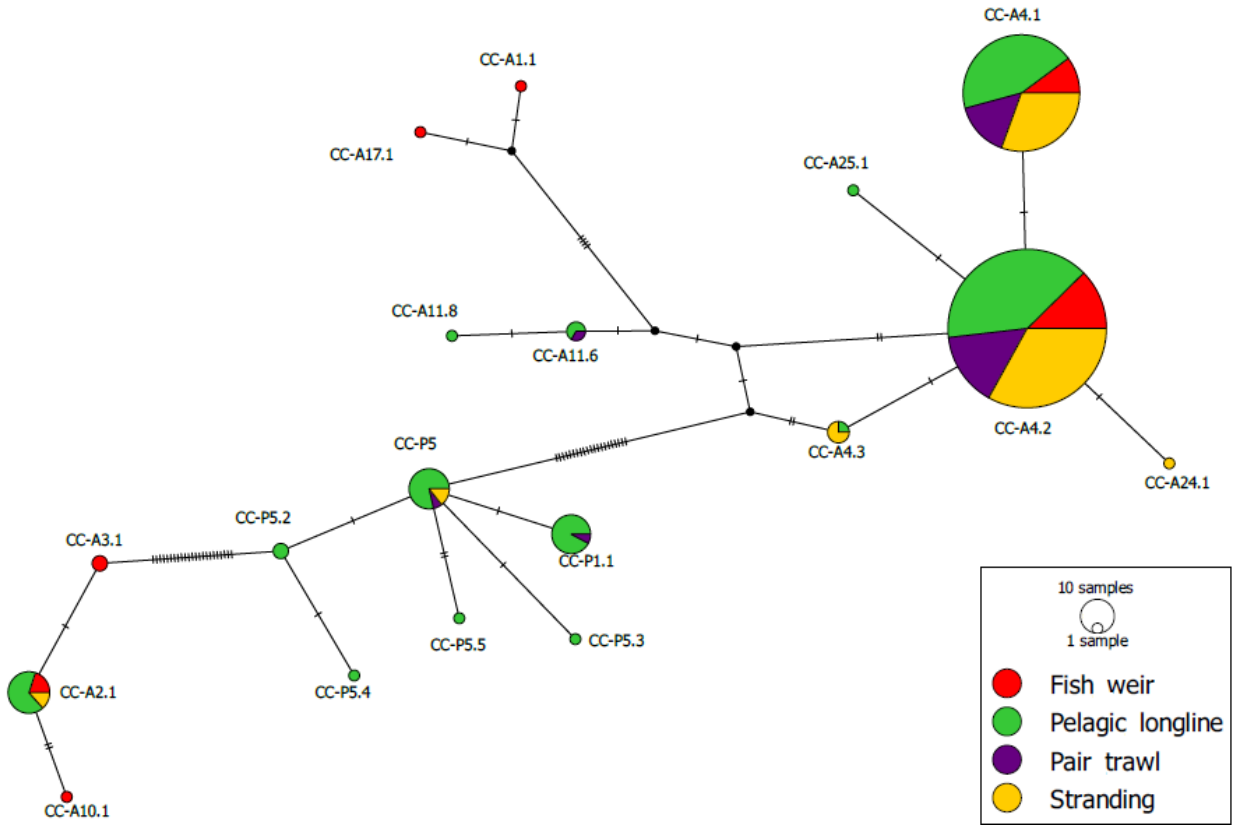
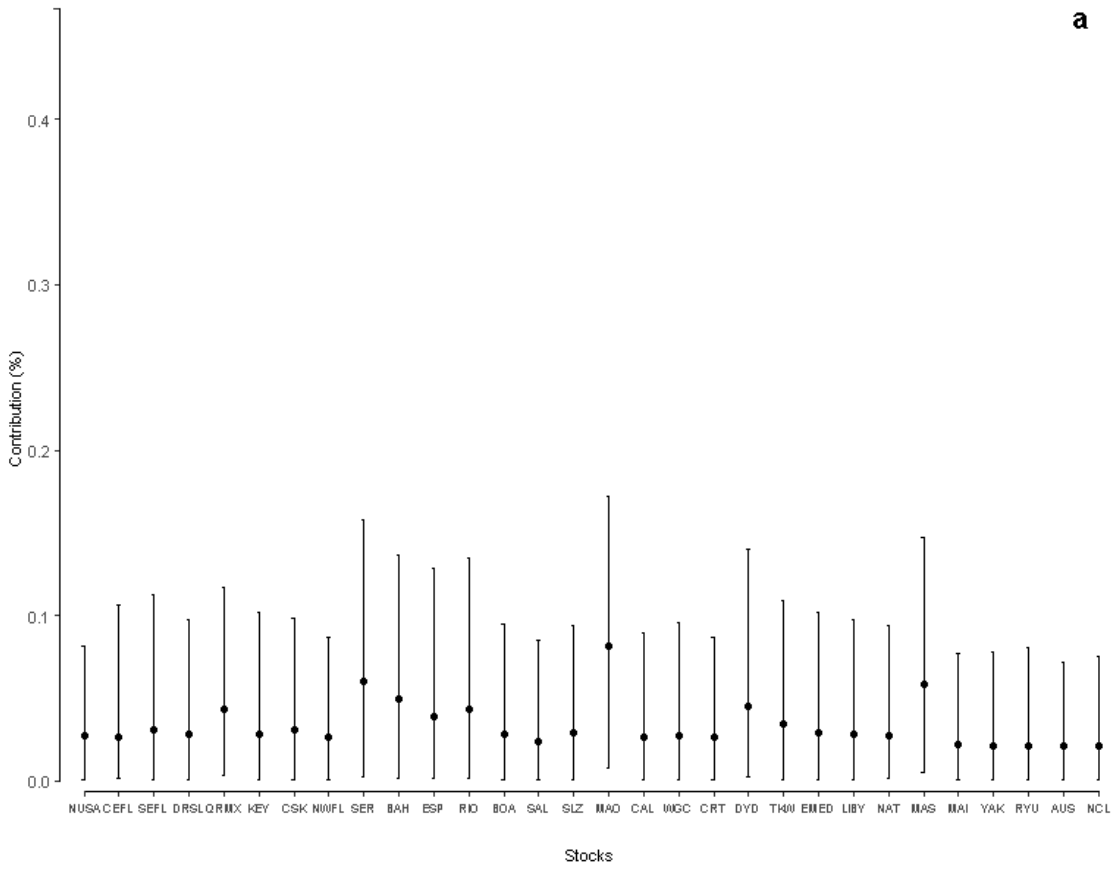
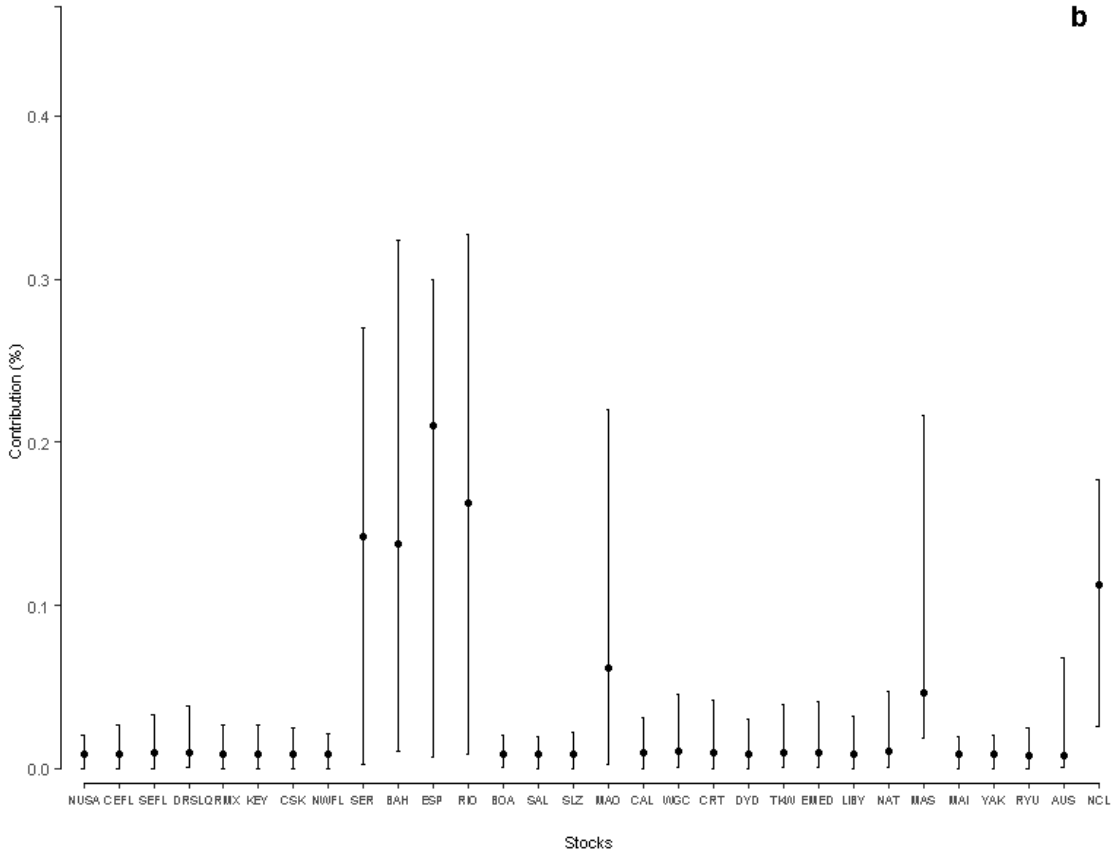
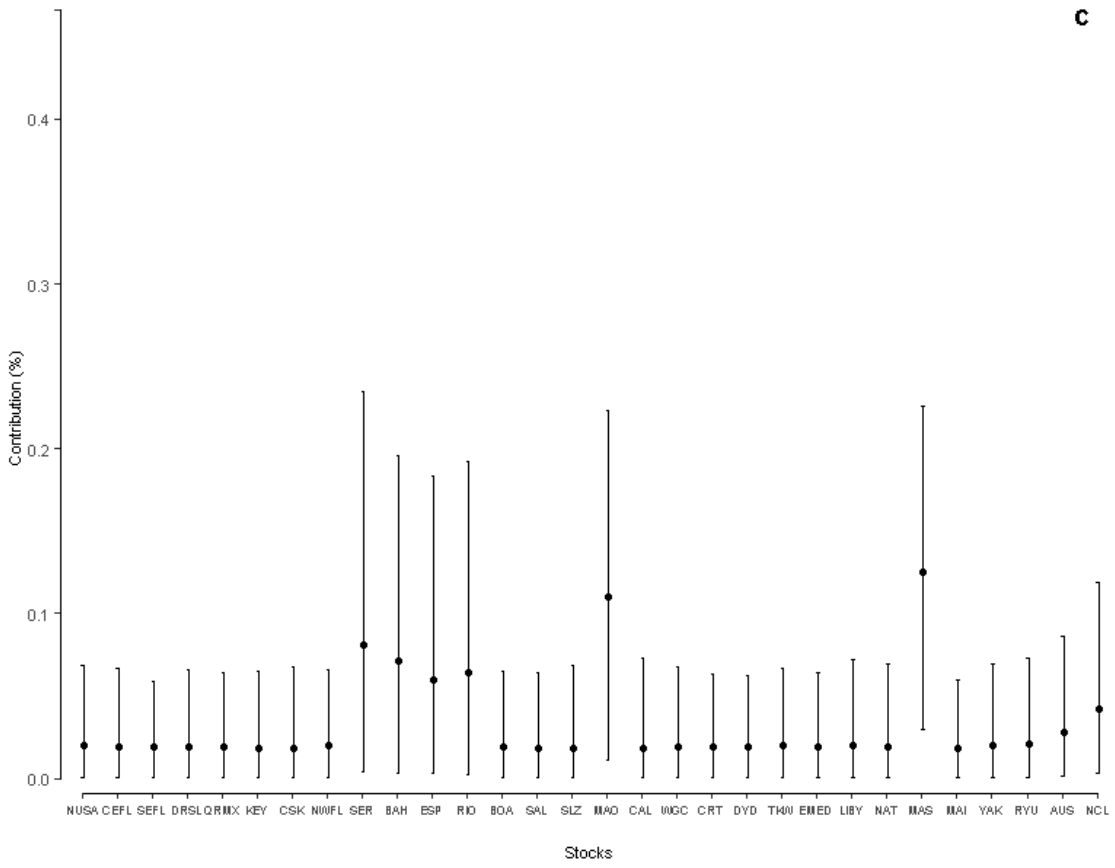

CC-A4.1
CC-A1.1
CC-A17.1
CC-A25.1
CC-A11.8
CC-A11.6
CC-A4.2
CC-A4.3
CC-A24.1
CC-P5
CC-P5.2
CC-A3.1
CC-P1.1
CC-P5.5
CC-P5.3
CC-A2.1
CC-P5.4
CC-A10.1
10 samples
1 sample
Fish weir
Pelagic longline
Pair trawl
Stranding

Fig. 4.


a
Contribution (%)
0.0
0.1
0.2
0.3
0.4
NUSA CEFL SEFL DRSL QRMX KEY CSK NWFL SER BAH BSP RIO BOA SAL SIZ MAO CAL WGC CRT DYD TWN EMED LIBY NAT MAS MAI YAK RYU AUS NCL
Stocks


b
Contribution (%)
0.0
0.1
0.2
0.3
0.4
NUSA CEFL SEFL DRSL QRMX KEY CSK NWFL SER BAH BSP RIO BOA SAL SIZ MAO CAL WGC CRT DYD TWN EMED LIBY NAT MAS MAI YAK RYU AUS NCL
Stocks


c
Contribution (%)
0.0
0.1
0.2
0.3
0.4
NUSA CEFL SEFL DRSL QRMX KEY CSK NWFL SER BAH BSP RIO BOA SAL SLZ MAO CAL WGC CRT DYD TKW EMED LIBY NAT MAS MAI YAK RYU AUS NCL
Stocks

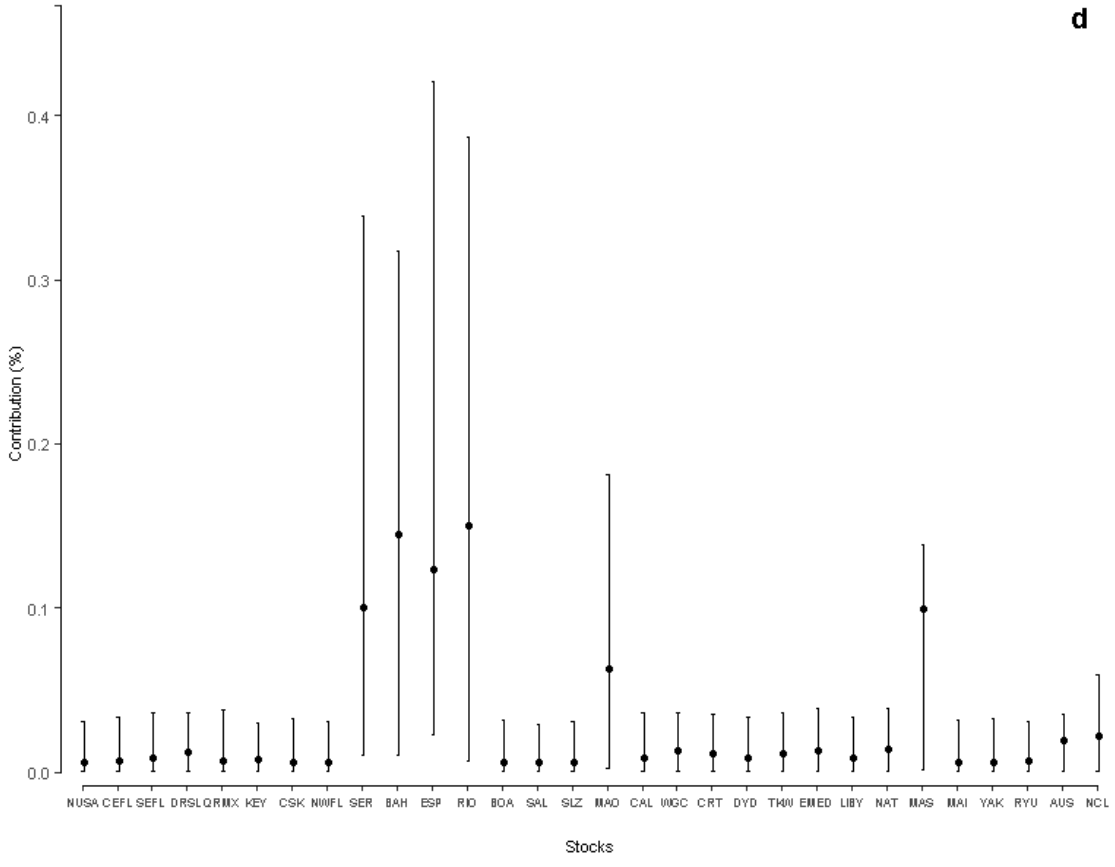

d
Contribution (%)
0.0
0.1
0.2
0.3
0.4
NUSA CEFL SEFL DRSL QRMX KEY CSK NWFL SER BAH BSP RIO BOA SAL SLZ MAO CAL WGC CRT DYD TKW EMED LIBY NAT MAS MAI YAK RYU AUS NCL
Stocks

Fig. 5.

Supplementary material

Table S1. Management units and nesting grounds of loggerheads sea turtles used in baseline of Mixed Stock Analysis.

Abbreviation	Rookery	Reference
NUSA	Cape Island, South Carolina, USA	Shamblin et al., 2014
	Ossabaw Island, Georgia, USA	Shamblin et al., 2014
CEFL	Canaveral National Seashore, Florida, USA	Shamblin et al., 2014
	Melbourne Beach, Florida, USA	Shamblin et al., 2014
SEFL	Juno Beach, Florida, USA	Shamblin et al., 2014
	Ft. Lauderdale, Florida, USA	Shamblin et al., 2014
DRSL	Cay Sal, Bahamas	Shamblin et al., 2014
	Drt Tortugas, Florida, USA	Shamblin et al., 2014
QRMX	Isla Cozumel, Quintana Roo, Mexico	Shamblin et al., 2014
	Quintana Roo mainland, Mexico	Shamblin et al., 2014
KEY	Keewaydin Island, Florida, USA	Shamblin et al., 2014
CSK	Casey Key, Florida, USA	Shamblin et al., 2014
NWFL	St. George Island, Florida, USA	Shamblin et al., 2014
	Cape San Blas, Florida, USA	Shamblin et al., 2014
SER	Sergipe, Brazil	Shamblin et al., 2014
BAH	Bahia, Brazil	Shamblin et al., 2014
ESP	Espírito Santo, Brazil	Shamblin et al., 2014
RIO	Rio de Janeiro, Brazil	Shamblin et al., 2014
BOA	Boa Vista, Cape Verde	Shamblin et al., 2014
SAL	Sal, Cape Verde	Shamblin et al., 2014
SLZ	Santa Luzia, Cape Verde	Shamblin et al., 2014
MAO	Maio, Cape Verde	Shamblin et al., 2014
CAL	Calabria, Italy	Shamblin et al., 2014
WGRC	Zakynthos Island, Greece	Shamblin et al., 2014
	Kyparissia, Greece	Shamblin et al., 2014
	Lakonikos, Greece	Shamblin et al., 2014
CRT	Rethymno, Crete	Shamblin et al., 2014
DYDL	Dalyan, Turkey	Shamblin et al., 2014
	Dalaman, Turkey	Shamblin et al., 2014
TKW	western Turkey	Shamblin et al., 2014
	middle Turkey	Shamblin et al., 2014
EMED	eastern Turkey	Shamblin et al., 2014
	Alagadi, Cyrpus	Shamblin et al., 2014
	Akamas, Cyprus	Shamblin et al., 2014
	El Mansouri, Lebanon	Shamblin et al., 2014
	Israel	Shamblin et al., 2014
LIBY	Sirte, western Libya	Shamblin et al., 2014
	Misurata, western Libya	Shamblin et al., 2014

NAT	Tongaland, KwaZulu-Natal, South Africa	Shamblin et al., 2014
MAS	Masirah, Oman	Shamblin et al., 2014
MAI	Bousou, mainland Japan	Matsuzawa et al., 2016
	Enshu-nada, mainland Japan	Matsuzawa et al., 2016
	Shikoku, mainland Japan	Matsuzawa et al., 2016
	eastern Kyushu, mainland Japan	Matsuzawa et al., 2016
YAK	Yakushima , Japan	Matsuzawa et al., 2016
RYU	Ryukyu Archipelago, Japan	Matsuzawa et al., 2016
AUS	Mon Repos, eastern Australia	Boyle et al., 2009
	Swain Reefs, eastern Australia	Boyle et al., 2009
	Wreck Island, eastern Australia	Boyle et al., 2009
	Wreck Rock, eastern Australia	Boyle et al., 2009
NCL	New Caledonia	Boyle et al., 2009

Table S2. Haplotype frequencies of management units and nesting grounds of loggerhead sea turtles used in Mixed Stock Analysis.

Haplotype	NUSA	CEFL	SEFL	DRSL	QRMX	KEY	CSK	NWFL	SER	BAH	ESP	RIO	BOA	SAL	SLZ	MAO	CAL	WGRC	CRT	DYDL	TKW	EMED	LIBY	NAT	MAS	MAI	YAK	RYU	AUS	NCL
CC-A1.1	141	668	27	1	0	10 6	25 0	94	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A1.2	0	15	1	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A1.3	0	3	16	1	1	0	2	0	0	0	0	0	84	32	19	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A1.4	0	20	7	1	13	0	7	2	0	0	0	0	8	3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A1.5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A1.7	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A1.8	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A2.1	0	275	12 3	46	64	80	10 4	12	0	0	0	0	2	2	0	0	28	67	16	30	60	194	23	98	0	0	0	0	0	0
CC-A2.2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A2.3	0	1	3	0	6	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A2.4	0	1	8	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A2.5	0	1	1	0	10	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A2.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0
CC-A2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	11	0	0	0	0	0	0	0
CC-A2.11	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A3.1	0	37	14	1	3	13	47	1	0	0	0	0	0	0	0	0	0	0	0	30	16	8	4	0	0	0	0	0	0	0
CC-A3.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0

CC-A4.1	0	0	0	0	0	0	0	0	10	40	13	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A4.2	0	0	0	0	0	0	0	0	23	26	31	65	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A4.3	0	0	0	0	0	0	0	0	0	0	4	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A5.1	0	2	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A6.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A7.1	0	7	2	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A7.2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A8.1	0	0	0	0	7	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A9.1	0	2	2	2	8	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A10.1	0	9	7	0	41	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A10.4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A11.2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A11.3	0	0	0	1	7	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A11.5	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A11.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0
CC-A12.1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A13.1	0	1	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
CC-A14.1	0	15	1	0	7	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A17.1	0	0	0	0	0	0	0	0	0	0	0	0	41	5	13	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

CC-A17.2	0	0	0	0	0	0	0	0	0	0	0	0	2	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A20.1	0	2	2	0	0	5	1	0	0	0	0	0	0	0	0	0	17	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A21.1	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A24.1	0	0	0	0	0	0	0	0	5	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A26.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
CC-A27.1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A29.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
CC-A31.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A32.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A36.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A36.2	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A41.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A42.1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A43.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A47.1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A50.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
CC-A51.1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A52.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
CC-A53.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0

CC-A59.1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A60.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CC-A65.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
CCP1.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	34	101	27	
CCP2.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	189	78	62	0	0
CCP2.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	29	1	0	0	0
CCP2.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	44	3	30	0	0
CCP2.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
CCP2.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
CCP3.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	39	22	20	0	0
CCP3.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0
CCP5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	

Table S3. Estimated contributions of loggerhead rookeries to foraging aggregations in northern and southern Brazil, based on Bayesian Markov Chain Monte Carlo (MCMC) mixed stock analysis. Mean values are shown with S.D. The 2.5 and 97.5% values indicate the upper and lower bounds of the 95% credibility interval.

	Fish weir				Pelagic longline				Bottom pair trawl				Strandings			
Stocks	Mean	S.D.	2.5%	97.5%	Mean	S.D.	2.5%	97.5%	Mean	S.D.	2.5%	97.5%	Mean	S.D.	2.5%	97.5%
NUSA	0.027	0.02255	0.00073	0.08171	0.009	0.00589	0.00015	0.02085	0.020	0.01857	0.00048	0.06813	0.006	0.00871	0.00029	0.03078
CEFL	0.026	0.02827	0.00111	0.10630	0.009	0.00688	0.00013	0.02632	0.019	0.01864	0.00038	0.06704	0.007	0.00906	0.00024	0.03331
SEFL	0.031	0.02791	0.00080	0.11280	0.010	0.00901	0.00019	0.03280	0.019	0.01643	0.00040	0.05863	0.009	0.00971	0.00027	0.03609
DRSL	0.028	0.02529	0.00040	0.09762	0.010	0.01065	0.00032	0.03849	0.019	0.01826	0.00044	0.06589	0.012	0.00979	0.00020	0.03586
QRMX	0.043	0.03122	0.00285	0.11720	0.009	0.00734	0.00021	0.02663	0.019	0.01732	0.00045	0.06432	0.007	0.01036	0.00034	0.03782
KEY	0.028	0.02784	0.00080	0.10170	0.009	0.00735	0.00022	0.02671	0.018	0.01891	0.00059	0.06473	0.008	0.00878	0.00038	0.03034
CSK	0.031	0.02853	0.00065	0.09818	0.009	0.00643	0.00016	0.02516	0.018	0.01846	0.00045	0.06767	0.006	0.00891	0.00019	0.03299
NWFL	0.026	0.02379	0.00072	0.08716	0.009	0.00548	0.00023	0.02100	0.020	0.01838	0.00051	0.06564	0.006	0.0086	0.00021	0.03049
SER	0.060	0.04181	0.00263	0.15780	0.142	0.07447	0.00297	0.27040	0.081	0.06476	0.00358	0.23500	0.100	0.08895	0.01069	0.33920
BAH	0.049	0.03708	0.00144	0.13630	0.138	0.08721	0.01037	0.32390	0.071	0.05239	0.00313	0.19550	0.145	0.08444	0.01025	0.31780
ESP	0.039	0.03509	0.00126	0.12830	0.210	0.07886	0.00708	0.30000	0.060	0.04992	0.00271	0.18340	0.123	0.10451	0.02274	0.42060
RIO	0.043	0.03671	0.00135	0.13450	0.163	0.0852	0.00856	0.32710	0.064	0.05245	0.00231	0.19240	0.150	0.10154	0.00707	0.38700
BOA	0.028	0.02544	0.00093	0.09449	0.009	0.00574	0.00029	0.02084	0.019	0.0169	0.00050	0.06504	0.006	0.00824	0.00027	0.03134
SAL	0.024	0.02336	0.00045	0.08456	0.009	0.00569	0.00012	0.01991	0.018	0.01713	0.00042	0.06398	0.006	0.00842	0.00022	0.02932
SLZ	0.029	0.02532	0.00088	0.09343	0.009	0.00593	0.00013	0.02247	0.018	0.01945	0.00033	0.06811	0.006	0.00857	0.00022	0.03050
MAO	0.081	0.04276	0.00727	0.17200	0.062	0.06263	0.00243	0.21990	0.110	0.05639	0.01099	0.22300	0.063	0.04928	0.00252	0.18120
CAL	0.026	0.02433	0.00057	0.08980	0.010	0.00866	0.00023	0.03126	0.018	0.01952	0.00042	0.07312	0.009	0.00934	0.00028	0.03579
WGRC	0.027	0.02514	0.00065	0.09536	0.011	0.01219	0.00030	0.04563	0.019	0.01829	0.00050	0.06742	0.013	0.00967	0.00031	0.03584
CRT	0.026	0.02416	0.00053	0.08662	0.010	0.01099	0.00026	0.04183	0.019	0.01755	0.00050	0.06354	0.011	0.00975	0.00033	0.03575
DYDL	0.045	0.03622	0.00247	0.14010	0.009	0.00842	0.00024	0.03028	0.019	0.0173	0.00055	0.06254	0.009	0.00913	0.00024	0.03345
TKW	0.034	0.03087	0.00096	0.10920	0.010	0.0101	0.00029	0.03877	0.020	0.01824	0.00071	0.06641	0.011	0.01004	0.00036	0.03644

EMED	0.029	0.02737	0.00080	0.10210	0.010	0.01115	0.00029	0.04081	0.019	0.01719	0.00063	0.06447	0.013	0.01031	0.00023	0.03867
LIBY	0.028	0.02751	0.00085	0.09737	0.009	0.00863	0.00023	0.03209	0.020	0.01829	0.00053	0.07226	0.009	0.00921	0.00027	0.03334
NAT	0.027	0.02499	0.00109	0.09377	0.011	0.01296	0.00037	0.04728	0.019	0.01855	0.00048	0.06934	0.014	0.00998	0.00029	0.03876
MAS	0.058	0.03751	0.00475	0.14710	0.046	0.05551	0.01856	0.21660	0.125	0.05269	0.02924	0.22590	0.099	0.03928	0.00124	0.13900
MAI	0.022	0.02109	0.00050	0.07727	0.009	0.00535	0.00016	0.01959	0.018	0.01725	0.00051	0.05972	0.006	0.00884	0.00019	0.03160
YAK	0.021	0.02091	0.00052	0.07780	0.009	0.00586	0.00013	0.02076	0.020	0.01819	0.00055	0.06920	0.006	0.00875	0.00021	0.03279
RYU	0.021	0.02115	0.00070	0.08066	0.008	0.007	0.00018	0.02465	0.021	0.01907	0.00062	0.07324	0.007	0.00831	0.00032	0.03058
AUS	0.021	0.01983	0.00054	0.07169	0.008	0.01857	0.00029	0.06769	0.028	0.02392	0.00091	0.08594	0.019	0.00984	0.00018	0.03522
NCL	0.021	0.01947	0.00049	0.07548	0.113	0.03744	0.02598	0.17720	0.042	0.03112	0.00298	0.11860	0.022	0.01603	0.00099	0.05921

CAPÍTULO 2

Origin and foraging ecology of male loggerhead sea turtles from southern Brazil revealed by genetic and stable isotope analysis

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Origin and foraging ecology of male loggerhead sea turtles from southern Brazil revealed by genetic and stable isotope analysis

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Abstract

The southwestern Atlantic Ocean (SWA) represents an important foraging ground for loggerhead sea turtles (*Caretta caretta*). Most studies at the region have focused on adult females and juveniles, and little is known about males. Here, we present the first insights about origin and foraging ecology of male loggerheads from the SWA, by integrating genetic and stable isotope analysis (SIA). Skin samples were obtained from 26 males stranded along the southern coast of Brazil (from 31°21'S–51°05'W to 33°44' S–53°22' W), from February 2014 to March 2017. Samples of potential food sources (benthic and pelagic organisms) were also collected for SIA. A fragment of the mitochondrial DNA control region was sequenced and a Bayesian Mixed Stock Analysis was performed to estimate natal origins of male loggerheads. Bayesian Stable Isotope Mixing Models were fitted to assess the relative contribution of different food sources assimilated by males.

Most males exhibit endemic haplotypes from Brazilian rookeries, followed by a low frequency of a haplotype from the North Atlantic and the Mediterranean Sea, as well as olive ridley (*Lepidochelys olivacea*) haplotype, showing hybridization. SIA showed life stage-related differences in feeding and habitat use by male loggerheads, with benthic invertebrates dominating the diet of adults, while pelagic prey items dominated the diet of juveniles. Our findings demonstrate the importance of southern Brazil neritic and oceanic habitats for male loggerheads and highlight the value of this area for the maintenance of SWA reproductive management units, which are the main contributors to these feeding aggregations.

Introduction

The loggerhead sea turtle *Caretta caretta* has a worldwide tropical and subtropical distribution, but global numbers have been reduced over the last decades due to extensive fishing-related mortality (Wallace et al. 2013). Sea turtles are particularly sensitive to population reductions, especially to mortality of large juveniles and adults, because they are slow-growing and late-breeding animals (Petitet et al. 2012; Avens et al. 2015). Although the abundance of several populations of loggerhead sea turtles is currently stable or increasing, this species is classified as *Vulnerable* in the IUCN Red List and is considered entirely conservation-dependent (Rees et al. 2016; Casale and Tucker 2017).

Similar to others sea turtle species, loggerhead turtles exhibit complex life histories that encompass migrations between nesting and feeding grounds, ontogenetic shifts in diet and habitat use, and natal homing (Bolten 2003; Jensen et al. 2013). A long-standing paradigm predicates that early juveniles spend their first years in the oceanic habitats (~ 12 years for southwestern Atlantic populations; Petitet et al. 2012), feeding opportunistically on pelagic prey items, followed by a period where loggerheads supposedly recruit to neritic habitats where they remain until adulthood, feeding preferentially upon benthic invertebrates (Bjorndal 1997; Bolten 2003; Barros 2010). After reaching sexual maturity at about 30 years of age (Petitet et al. 2012), adult females and males display asynchronous seasonal migrations between foraging and nesting grounds, the latter located at their natal areas (FitzSimmons et al. 1997; Plotkin 2003). However, studies indicate that this generalized life history model is not a rule and that interindividual variation in foraging and migratory behaviour is displayed by juveniles and adult loggerheads among ocean basins (Hatase et al. 2002b; Casale et al. 2007;

Mansfield et al. 2009; McClellan et al. 2010; Vander-Zanden et al. 2010; Zbinden et al. 2011). Factors that cause these variations remain poorly understood but could be related to sea surface temperature (Mansfield et al. 2009; Monteiro 2017), resource availability (Pajuelo et al. 2016) and phenotypic plasticity (Hawkes et al. 2006; Watanabe et al. 2011).

Knowledge of ecological features (e.g. habitat and resource use, migratory routes, and connectivity among nesting and foraging grounds) are essential for the development and application of appropriate conservation measures for sea turtle populations (Rees et al. 2016). Stable isotope analysis (SIA) has been extensively applied in ecological research of sea turtles, providing valuable information about diet resources (Dodge et al. 2011), habitat use (Reich et al. 2010; Petitet and Bugoni 2017), and ontogenetic shifts (Arthur et al. 2008; Snover et al. 2010). Stable isotope measurements of nitrogen (δ^{15}N) can be used to assess the trophic level of a consumer (DeNiro and Epstein 1981; Minagawa and Wada 1984), whereas stable isotope values of carbon (δ^{13}C) are often used to discriminate habitat use (e.g. neritic *versus* oceanic) and food sources (Fry 2006). Stable isotope values in consumers reflect those of prey items within a timescale that depends on the tissue's turnover rate, which is a species-specific trait (Peterson and Fry 1987; Fry 2006). Therefore, tissue selection is an important aspect for dietary reconstruction (Perkins et al. 2013). In loggerheads, skin provides dietary isotopic signatures over a time frame of ~45 days (Reich et al. 2008) without being modified with the specimen's decomposition (Payo-Payo et al. 2013).

In recent years, studies using SIA and satellite telemetry have revealed a wide variety of movements, habitat use and foraging strategies of loggerhead adult males and females, as well as juveniles, over ocean basins (Hatase et al. 2002b; Hawkes et al. 2006; McClellan et al. 2010; Pajuelo et al. 2012; Pajuelo et al. 2016). These studies have reported an extensive foraging dichotomy in loggerhead populations, which in some cases were related to turtle sizes. In the case of females, a similar pattern was observed in the western Pacific and eastern Atlantic Ocean, where larger adult individuals preferentially use neritic habitats, while smaller ones occurred in oceanic habitats (Hatase et al. 2002b; Hawkes et al. 2006). For adult male loggerheads from the North Pacific, differences in feeding habitat appeared to also be influenced by body size. While larger males remained in coastal waters (Saito et al. 2015), smaller males inhabited pelagic habitats (Hatase et al. 2002a). In the Mediterranean Sea, however, although polymorphisms in habitat use were observed in adult male loggerheads, the variability in feeding behaviours was not

associated with size (Schofield et al. 2010). Moreover, previous studies showed that adult males can display residency behaviour around breeding areas and fidelity to foraging grounds in northwestern and southeastern Atlantic waters (Arendt et al. 2012; Varo-Cruz et al. 2013) and in the Mediterranean Sea (Schofield et al. 2010; Casale et al. 2013), and that some degree of individual specialization can be shown according to resource availability (Pajuelo et al. 2016). Despite the increasing knowledge in different ocean basins, no information is available about the spatial ecology and feeding behaviour of male loggerhead sea turtles in the southwestern Atlantic Ocean (SWA).

The SWA is an important habitat for loggerheads, with several significant nesting and feeding grounds (Marcovaldi and Marcovaldi 1999; Vélez-Rubio et al. 2013; Carman et al. 2016; Monteiro et al. 2016), but also harboring extensive fishery activities that overlap with sea turtle distributions (Sales et al. 2008; Wallace et al. 2013; Monteiro et al. 2016). This high overlap increases the probability of turtle bycatch and is the main source of the thousands of dead loggerhead strandings in southern Brazil over the last twenty years (Monteiro et al. 2016). However, the effects of this mortality on the demographic and genetic structure of loggerhead populations remain unclear. The Brazilian rookeries host one of the largest numbers of nests in the world (ca. 7000 to 8000 nests/year; Marcovaldi et al. 2018), with nesting grounds ranging over a wide latitudinal area from Sergipe (northeastern) to Rio de Janeiro (southeastern) (Fig. 1; Marcovaldi and Chaloupka 2007). Satellite tracking data of adult female loggerheads from the Bahia nesting population revealed high fidelity to foraging grounds in the Brazilian coast during breeding and post-breeding periods. The movements between nesting and foraging grounds occurred along the continental shelf, where the northern coast of Brazil stood out as the most important foraging ground for reproductive females (Marcovaldi et al. 2010). A phylogeographic study using mitochondrial DNA (mtDNA) have recognized globally, 18 demographically independent management units (MUs – *sensu* Moritz 1994) for loggerhead sea turtles (Shamblin et al. 2014). MUs are defined as rookeries with significant genetic distinction, and can be defined by differences in mtDNA haplotype frequencies (Moritz 1994). Brazilian rookeries hold endemic haplotypes, which provide a unique profile (Reis et al. 2010b), and were recognized as three MUs within the SWA: (1) the northeastern coast (Sergipe and Bahia), (2) Espírito Santo, and (3) Rio de Janeiro (Shamblin et al. 2014). The genetic differentiation of nesting populations worldwide enables the estimation of the origin of turtles sampled in foraging grounds, which are

composed of individuals from multiple rookeries (i.e. mixed stocks), and offer great insights about the migratory behaviour and connectivity of sea turtle populations (Rees et al. 2017; Tolve et al. 2018; but see Prosdocimi et al. 2015). Southern Brazil is an important foraging ground for adults and juveniles of loggerheads in both neritic and oceanic habitats (Barros 2010; Monteiro et al. 2016). Oceanic feeding aggregations at the region are composed mainly by juvenile loggerheads from Brazilian rookeries, and in lower proportion by juveniles from North Atlantic, Mediterranean, and Pacific nesting grounds (Reis et al. 2010b; Shamblin et al. 2014). In the current study, we integrated SIA and genetic analysis of mtDNA to infer natal origins, movements and feeding ecology of male loggerhead sea turtles from southern Brazil foraging grounds and to provide first insights about the life history of these animals in the SWA.

Methods

Sample collection

Tissues samples were collected from male loggerhead sea turtles stranded dead on the coast of Rio Grande do Sul (RS), southern Brazil, between Lagoa do Peixe (31°21'S, 51°05'W) and Arroio Chuí (33°44' S, 53°22' W) (Fig. 1). Sampling took place mainly during austral summer of 2014 through 2017, but a few specimens were sampled in late spring, early autumn and early winter (Table S1). For each specimen, curved carapace length (CCL) was measured with a flexible metric tape (± 0.1 cm), from the midline of the nuchal notch to the posterior end of the posterior marginal scute (Bolten 1999). Sex was determined by the examination of gonads during necropsy (Wyneken 2001) and/or by tail length (for adults > 90 cm CCL; Wibbels 1999; Casale et al. 2005). The stranded specimens were also classified according to the decomposition state of carcasses, as follows: 1 = freshly dead (eyes present); 2 = initial decomposition (without eyes); 3 = moderate decomposition (swollen body, fluids apparent in the orifices, loss of carapace scutes and/or head scales); 4 = intermediate decomposition (shriveled body, carapace peeling, conspicuous bone plates); and 5 = advanced decomposition (carcass mummified). For genetic analysis, fragments of skin were collected and stored in absolute ethanol or DMSO solution. Skin samples obtained for SIA were stored in plastic bags and frozen at -20°C until laboratory procedures. Additionally, samples of potential food items of loggerhead sea turtles from neritic and oceanic environments, as observed by Bugoni et al. (2003) and Barros (2010), were also collected. These items included the

following taxonomic groups: the gastropod *Buccinanops monoliferum*, the squid *Dorytheuthis plei*, the hermit crabs *Dardanus insignis* and *Loxopagurus loxochelis*, the spider crab *Libinia spinosa*, salps (Class Thaliacea, Order Salpida, Family Salpidae), and the fishes whitemouth croaker *Micropogonias furnieri*, banded croaker *Paralonchurus brasiliensis* and cutlassfish *Trichiurus lepturus*. Anemones (Class Anthozoa, Order Actinaria) associated with hermit shells and the pelagic jellyfish *Lychnorhiza lucerna* (Class Scyphozoa, Order Rhizostomeae) were also collected. Cnidarians have no calcareous or chitinous structure (with exception of some hydrozoans such as *Velella velella*; Francis 1985) and are rapidly digested and difficult to detect in the gastrointestinal tract when consumed by sea turtles (Van Nierop and Hartog 1984). Although these taxa have not been observed in stomach contents of loggerhead sea turtles evaluated in southern Brazil (Bugoni et al. 2003; Barros 2010), they were reported as prey in the North Atlantic, Mediterranean Sea, and Australian coasts (Jones and Seminoff 2013). Crustacean, anemone, cephalopod and fish specimens were obtained from commercial fisheries that operate at the continental shelf adjacent to the study area. Salps were collected at the shelf-break and slope off southern Brazil, between the 550 and 3000 m depth isobaths on oceanographic cruises carried out onboard the R/V *Atlântico Sul* of the Universidade Federal do Rio Grande (FURG). The remaining prey species were collected during beach surveys conducted at the study area.

Genetic analysis

For genetic analysis, DNA was extracted from tissues with a PureLink™ Genomic DNA Kit. A fragment of 818 base-pairs (bp) of the mtDNA D-loop region was amplified through Polymerase Chain Reactions (PCR) with the primer pair LCM15382 (5- GCT TAA CCC TAA AGC ATT GG -3') and H950 (5- GTC TCG GAT TTA GGG GTT TG -3′) (Abreu-Grobois et al. 2006). PCR reactions contained 20 to 50 ng of genomic DNA, 5U of Platinum Taq Polymerase or Recombinant Taq Polymerase (Invitrogen 10966-030 and 11615-010, respectively), 0.2 µM of each primer, 0.4 mM of dNTPs, 1x PCR Buffer and 0.1 mM $MgCl_2$. Negative controls were included to detect possible contaminations during the amplification process. PCRs were performed with an Applied Biosystems Veriti 96-well Thermocycler under the following conditions: denaturation of 5′ at 94°C; 36 cycles of 30″ at 94ºC, 30″ at 50ºC, 1′ at 72ºC; and a final extension of 10′ at 72ºC. The obtained PCR products were purified with a PureLink™ Quick Gel Extraction and

Purification Combo Kit. Purified PCR products were sequenced in forward and reverse direction using a ABI PRISM 3730XL Analyzer.

Sequences were manually edited and aligned using the program BioEdit ver 7.0.9 (Hall 1999) and classified according to previously described haplotypes recorded in the Archie Carr Center for Sea Turtle Research database (http://accstr.ufl.edu/) and GenBank (http://ncbi.nlm.nih.gov). Throughout this paper we use standardized haplotype nomenclature established by both databases. The original haplotype denomination is based on short mtDNA fragments (380bp) and were recorded with subsequent numbers. Haplotypes based on the larger fragment (~800 bp) remained with their original short sequence designations, but receive numeral suffixes to indicate polymorphisms observed within the expanded sequences (Shamblin et al. 2014). Haplotype (h) and nucleotide (π) diversities (Nei 1987) were calculated using the program DNAsp 5.10 (Rozas et al. 2003). There is little information on standard diversity indices based on long sequences of loggerheads sea turtles, making it difficult to compare with previous studies carried out in loggerhead foraging grounds in the Atlantic. Thus, here we calculated haplotype and nucleotide diversities for short and long sequences. A *'many-to-one'* Bayesian Mixed Stock Analysis (MSA) was performed in order to estimate the natal origins of male loggerhead sea turtles (Pella and Masuda 2001). MSA was carried out using R software version 3.4.2 (R Core Team 2017) through the package *mixstock*, which estimates the contribution from source populations to one or more mixed stocks (Bolker et al. 2007). As a baseline, we used the haplotype frequencies matrix of loggerhead rookeries provided by Shamblin et al. (2014). A Markov Chain Monte Carlo (MCMC) was applied to obtain the posterior distributions of stocks contributions, integrating the data likelihood with an uninformative prior, through four chains of 20,000 iterations with an initial discard of the first 10,000, resulting in posterior distributions with 95% credibility intervals (CrI 95%). The Gelman-Rubin reduction factor was used to verify convergence of chains by comparing variances between them. Values below 1.2 for all parameters indicate that convergence was achieved and the corresponding estimates are reliable (Bolker et al. 2007).

MSA is a powerful tool to understand the migration patterns of sea turtles, but the frequency of some haplotypes in sources populations can affect the model fit, providing results with large uncertainty. The occurrence of common haplotypes that are shared among rookeries but are rare in mixed stocks results in contribution estimates that are

biologically unreliable (e.g. haplotype CC-A2 and variants, reported for the northwestern Atlantic, Mediterranean and South Africa loggerhead rookeries; Jensen et al. 2013; Shamblin et al. 2014). Previous studies suggested the exclusion of nesting grounds that increase error and generate unlikely stock contributions (Engstrom et al. 2002; Rees et al. 2017). To perform MSA, hybrid individuals should also be excluded because they represent orphaned haplotypes due to the lack of baseline data of hybrid haplotypes in Atlantic rookeries (Bolker et al. 2007).

Stable isotope analysis

Male loggerhead skin and prey tissues were rinsed with distilled water, oven dried to a constant mass at 60°C for 48 to 72 h, and powdered using a mortar and pestle. About 0.7 mg of each sample was weighed and placed in individual 4 x 6 mm tin cups. The prey tissues processed for SIA were: muscle from crustaceans and fishes, the longitudinal muscle of the column of anemones, the bell of jellyfishes, the mantle of gastropods and cephalopods, and the whole body of salps.

Lipid content of a tissue is a potential confounding factor in SIA, particularly in studies using mixing models (Kiljunen et al. 2006). In general, tissues that exhibit C:N ratios higher than 3.5 have lipid content with the potential to alter $\delta^{13}C$ values (Post et al. 2007). In such cases, lipid extraction or mathematical normalization is required (Post et al. 2007; Logan et al. 2008; Petitet and Bugoni 2017). According to previous studies carried out with green turtles *Chelonia mydas*, C:N ratios of sea turtles' skin are below the threshold of 3.5 and lipid content of this tissue does not appear to alter $\delta^{13}C$ values (Vander Zanden et al. 2012; Bergamo et al. 2016). For this reason, most skin samples were analyzed without lipid extraction. However, six adult males presented C:N ratios > 3.5, indicating high lipid content that could alter $\delta^{13}C$ values. Therefore, these skin samples were lipid-extracted using a Soxhlet apparatus with a 2:1 solvent mixture of chloroform and methanol for 6 h (Medeiros et al. 2015), and re-analyzed. Regarding prey samples, only anemones showed evidence of high lipid content. In this case, we normalized carbon isotopic values according to D'Ambra et al. (2014).

Samples of male loggerhead turtles, jellyfishes, spider crabs and gastropods were analyzed using a Costech 4010 elemental analyzer coupled to a Thermo Scientific Delta V isotope ratio mass spectrometer at the University of New Mexico Center for Stable

Isotopes (UNM–CSI). For the remaining samples, SIA was performed at the Stable Isotope Core Laboratory, Washington State University (SICL–WSU), with an elemental analyzer Costech 4010 connected to a Delta PlusXP isotope ratio mass spectrometer Thermofinnigan. Stable isotope values are expressed in δ-notation as parts per thousand (‰) differences from the international standard material, Vienna Pee Dee Belemnite limestone and atmospheric nitrogen (Air) for carbon and nitrogen, respectively, according to the following equation (as in Bond and Hobson 2012):

$$\delta X = (R_{sample}/R_{standard}) - 1 \quad (1)$$

where X is the ^{13}C or ^{15}N value, and R is the corresponding ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$ (Peterson and Fry 1987). Both laboratories use internal standards of known carbon and nitrogen composition in order to estimate instrument precision. The analytical precision (standard deviation – SD) of the internal laboratory standards used by UNM-CSI was measured at < 0.2‰ for $\delta^{15}N$ and < 0.04‰ for $\delta^{13}C$; for SICL–WSU the SD was < 0.1‰ for both $\delta^{13}C$ and $\delta^{15}N$.

Samples analyzed in different laboratories can be compared directly only after a calibration procedure. Isotopic values of feathers of the yellow-nosed albatross *Thalassarche chlororhynchos* revealed significant differences in $\delta^{13}C$ values between paired-samples analyzed both UNM–CSI and SICL–WSU laboratories (Leal 2018). Therefore, $\delta^{13}C$ values of our samples analyzed in SICL–WSU were corrected using following equation:

$$\delta^{13}C_{corrected} = (-1.59) + 0.92\ (\delta WSU) \quad (2)$$

where δWSU represents the $\delta^{13}C$ values obtained at the Stable Isotope Core Laboratory, Washington State University (Leal 2018).

A paired *t*-test was also conducted to verify significant differences between lipid-extracted and non-extracted skin samples, separately for $\delta^{13}C$ and $\delta^{15}N$ values. A general linear model (GLM) was fitted to verify significant differences in stable isotope values among size classes (i.e. adults and juveniles) and genetic composition (i.e. haplotype classification) of male loggerheads. Individual turtles were considered juveniles if CCL was smaller than the minimum maturation size (88.2 cm CCL) estimated for male

loggerheads from the North Atlantic (Avens et al. 2015). The GLM was fitted with a Gamma distribution, where $\delta^{13}C$ and $\delta^{15}N$ values were the response variable, and size classes and haplotypes were the categorical explanatory variables with two and five levels, respectively. The model fit was verified through residual diagnostics (e.g. quantile-quantile plots and Cook's distance). The variables that significantly affected $\delta^{13}C$ and $\delta^{15}N$ values were used to separate male loggerheads in different groups. The significance level for both paired *t*-test and GLM was $\alpha = 0.05$.

Stable Isotope Mixing Models (SIMM) integrate variability in resource and consumer isotope values, providing a framework for understanding trophic ecology (Parnell et al. 2013). For each group defined through the GLM, we estimated the relative contribution of potential prey to the diet of male loggerheads by fitting a Bayesian mixing model with the package *simmr* (Parnell 2016) developed for software R. Before running SIMM, we assessed three trophic discrimination factors (TDFs) provided by previous studies, as well the feasibility of the prey database using simulated mixing polygons with a Bayesian statistical framework through the packages *sp* and *splancs* (Smith et al. 2013). The mixing polygon simulation is visualized with a mixing region (i.e. convex hull), which is calculated by testing a grid of values for point-in-polygon bounded by the proposed food sources, providing a quantitative basis for model rejection, consumer exclusion (those outside the 95% mixing region) and the evaluation of the most appropriate TDFs (Smith et al. 2013). One set of TDFs is derived from a study carried out with captive early juvenile loggerhead sea turtles (Reich et al. 2008), which estimated the mean values (±SD) of residence time and TDF of several tissues. For skin, the estimated residence time was 46.1 ± 8.9 days for $\delta^{13}C$ and 44.9 ± 3.1 days for $\delta^{15}N$. The TDF was 1.11 ± 0.17‰ for $\delta^{13}C$ and 1.60 ± 0.07‰ for $\delta^{15}N$. The other two TDFs applied in mixing polygons simulations consisted of values estimated for skin of captive large juveniles and adults of green turtles from Cayman Turtle Farm in Grand Cayman, British West Indies (Vander Zanden et al. 2012). The estimated values for juveniles were 1.87 ± 0.56‰ for $\delta^{13}C$ and 4.77 ± 0.40‰ for $\delta^{15}N$, and for adults 1.62 ± 0.61‰ for $\delta^{13}C$ and 4.04 ± 0.04‰ for $\delta^{15}N$. After running SIMM, a diagnostic matrix plot was produced, providing the correlation among sources. This is a useful tool to identify when the model is fitting well, indicated by low correlations between sources, or when the model is unable to differentiate food items (Parnell 2016). Based on that, some prey species can be grouped according to the similarity in their isotope signatures, taking into account their habitat and

resource use preferences (Phillips et al. 2005). Due to the similarity in $\delta^{13}C$ and $\delta^{15}N$ values of crustaceans *L. spinosa* and *D. insignis*, and the fishes *M. furnieri* and *P. brasiliensis,* provided by the diagnostic plots of SIMM, we lumped these prey items in two groups, hereafter called as crabs and croakers, respectively. All statistical analyses involving isotopic values were carried out using R software version 3.4.2 (R Core Team 2017).

Results

A total of 26 male loggerhead sea turtles were sampled, of which 19 were adults and 7 were juveniles (Table S1). The CCL of all specimens ranged from 24 to 114 cm (mean = 88.8 ± 21.9 cm). One adult male could not be measured because the carapace was damaged by the carcass decomposition process. However, it was possible to infer that it was an adult male due to the large body size and long tail.

Genetic analysis

A total of five haplotypes were identified: CC-A2.1 (n = 1), CC-A4.1 (n = 7), CC-A4.2 (n = 15), CC-A4.3 (n = 1) and one haplotype typical of olive ridley (*Lepidochelys olivacea*) sea turtles (n = 2), haplotype F (hereafter referred to as Cc × Lo haplotype). Haplotype F was identified through the short sequence (380pb), since genetic characterization with long sequences has not been conducted for olive ridley rookeries in the Atlantic Ocean. Haplotype and nucleotide diversities are summarized in Table 1.

A preliminary MSA including all previously described rookeries provided unrealistic contribution estimates due to the presence of one male containing CC-A2.1 in our samples (data not shown). Therefore, a second MSA was performed removing the male with CC-A2.1 haplotype and the north Atlantic and Mediterranean nesting areas, resulting in a matrix only with Brazilian lineages. This second MSA showed that males in southern Brazil originated in a slightly greater proportion from Espírito Santo state, followed by Rio de Janeiro, Bahia and Sergipe states (Table 2).

Stable isotope analysis

Lipid-extracted and non-extracted skin samples from males differed significantly in $\delta^{13}C$ values (t = -5.64, P < 0.01), but not for $\delta^{15}N$ values (t = 0.64, P = 0.54). After lipid extraction procedures, all skin samples had C:N ratios <3.5 (Table S1). All males showed $\delta^{13}C$ values ranging from -18.01 to -12.99‰ (-14.73 ± 1.30‰) and $\delta^{15}N$ values ranging from 6.80 to 18.59‰ (14.77 ± 2.99‰). In adult males, $\delta^{13}C$ values ranged from -16.44 to -12.99‰ (-14.71 ± 0.90‰) and $\delta^{15}N$ values ranged from 10.69 to 18.59‰ (15.96 ± 1.69‰). In juveniles, $\delta^{13}C$ values ranged from -18.01 to -14.54‰ (-16.19 ± 1.13‰) and $\delta^{15}N$ values ranged from 6.80 to 14.26‰ (11.16 ± 3.02‰). $\delta^{13}C$ and $\delta^{15}N$ values were significantly lower in juveniles than adult males. The model also indicated a significant difference of $\delta^{15}N$ values of male hybrids compared to male with loggerhead haplotypes only (Table S2). Based on GLM results, we performed the mixing polygon simulations and the SIMM for adults and juveniles, separately. For adults, the mixing polygon simulation revealed that the most suitable TDFs for mixing models were the estimated values for adults of green sea turtles (Fig. 2b). The convex hull output also indicated that one adult individual was outside the 95% mixing region; therefore, this consumer was excluded from SIMM (Fig. 2b). For juveniles, the mixing polygon simulation revealed that the TDFs estimated for juvenile loggerheads was the most appropriate to use in our SIMM, and that one hybrid individual occurred outside the outermost contour of convex hull, thus being excluded from further analysis (Fig. 2c). Mixing polygon models using the other two sets of TDF values showed more individuals falling outside the convex hulls (Fig. 2a and d). Therefore, the two subsequent SIMMs were run with the sets of TDF values with the better fit for adults and juveniles, excluding one individual each (Table S1).

The best model fit consisted of nine food sources (values of potential food items are summarized in Table S3). Results from SIMM demonstrated greater contribution of the hermit *L. loxochelis* in the diet of adult male loggerheads (CrI 95% = 40.4 ± 18.4%, ranging from 3.9 to 70.8%; Fig. 3a), followed by the gastropod *B. monoliferum* (12.4 ± 10.2%, ranging from 1.1% to 39.5%). For the remaining food sources, the estimated contributions were homogeneous and close to zero. For juveniles, SIMM showed a greater contribution of salps (23.9 ± 11.3%, ranging from 3.1 to 45.8%; Fig. 3b), followed by the hermit *L. loxochelis* (13.9± 12.8%, ranging from 1.2 to 48.9%).

Discussion

The SWA represents an important foraging ground for loggerhead sea turtles at different life stages. However, most studies have focused on adult females and juveniles, and little is known about males. To the best of our knowledge, this study provides the first insights about the origins, movements and foraging ecology of male loggerhead sea turtles in the SWA, and highlights the usefulness of genetic and stable isotopes analysis as complementary methods to address life history features of sea turtles. Our study also describes for the first time hybridization in sea turtle males.

Origin and migrations

Genetic results showed that most males (88.5%) that occur in foraging grounds in southern Brazil exhibit haplotypes endemic from Brazilian rookeries (CC-A4.1, CC-A4.2 and CC-A4.3; Shamblin et al. 2014), accompanied by a low frequency of the haplotypes CC-A2.1 (3.8%) and Cc × Lo (7.7%). All adults sampled in our study were from Brazilian lineages. Haplotype CC-A2.1 (Genbank EU179445; Shamblin et al. 2012) is the most geographically widespread loggerhead sea turtle haplotype and is present in almost all of the western/eastern Atlantic and Mediterranean nesting grounds, except for those in Brazil (Shamblin et al. 2014). Haplotypes CC-A4.1, CC-A4.2 and CC-A4.3 (Genbank KF840723-25; Shamblin et al. 2014) are variants of the haplotype CC-A4 (Genbank AJ001077; Bolten et al. 1998), which is the ancestral haplotype exclusive to the Brazilian loggerhead populations (Reis et al. 2010b). Haplotype F, observed in hybrid males, has been recorded in olive ridley sea turtle rookeries from Brazil, Suriname and Guinea Bissau (Genbank AF051773; Bowen et al. 1998).

Analysis of the long mtDNA fragment greatly increased diversity estimates when compared to short haplotypes due to the higher proportion of polymorphic sites (Table 1). Based on the short mtDNA sequence, haplotype and nucleotide diversities found in males were lower than the ones found for unsexed turtles at oceanic feeding aggregations in southern Brazil ($h = 0.714 \pm 0.031$ and $\pi = 0.017 \pm 0.001$; Reis et al. 2010b) and foraging grounds in Uruguayan waters ($h = 0.431 \pm 0.087$ and $\pi = 0.014 \pm 0.007$; Caraccio et al. 2008), but higher than unsexed loggerheads from neritic aggregations of the Buenos Aires province, Argentina ($h = 0.032 \pm 0.031$ and $\pi = 0.000089 \pm 0.0003$; Prosdocimi et al. 2015). Previous studies that assessed the genetic composition of loggerhead sea turtles in foraging grounds in the SWA, without sexual differentiation, observed that coastal waters of Uruguay and Argentina are composed exclusively by adults and large juveniles from

Brazilian rookeries (Caraccio et al. 2008; Prosdocimi et al. 2015). In oceanic waters off Uruguay and Brazil, however, although Brazilian haplotypes are still the most frequent, foraging grounds are composed by smaller juveniles with higher genetic variability than those from neritic areas, in which haplotypes from the North Atlantic, Mediterranean Sea, and Pacific Ocean were reported (Caraccio et al 2008; Reis et al. 2010b).

Globally, hybridization events have been reported occasionally through genetic analysis (Karl et al. 1995; Seminoff et al. 2003; James et al. 2004). However, a high incidence of hybridization among Cheloniidae sea turtle species has been observed in Brazil (Lara-Ruiz et al. 2006; Reis et al. 2010a). The causes of this extensive hybridization are still unclear, but are likely a consequence of anthropogenic pressures that caused historic population declines and uneven sex ratios within populations and among species (Vilaça et al. 2012). The observed olive ridley haplotype is common at several rookeries in the Atlantic. However, to date, Cc × Lo hybrids have only been reported in the Sergipe rookery, possibly facilitated by the spatial and temporal overlap of the nesting seasons of both species (Reis et al. 2010a; Vilaça et al. 2012). Based on this, and considering the high frequency of endemic Brazilian haplotypes observed among males, we propose that hybrid males come from the Sergipe rookery.

MSA estimates indicate that male loggerheads from southern Brazil originated in a slightly greater proportion from nesting grounds in Espírito Santo, followed by Rio de Janeiro, Bahia and Sergipe rookeries (Fig. 1, Table 2). These results corroborate a sex ratio study that estimated that loggerhead sea turtle nests in Espírito Santo and Rio de Janeiro produce less female hatchlings than those in Sergipe and Bahia (Marcovaldi et al. 2016). While the northeastern nesting grounds produce a mean of 94% females (ranging from 83% to 99% in Sergipe, and from 79% to 98% in Bahia), at the southeastern region female offsprings correspond to a mean of 53% of nests (ranging from 33% to 81% in Espírito Santo and 18% to 81% in Rio de Janeiro). Sea turtle species have temperature-dependent sex determination, where the pivotal incubation temperature that leads to offspring with a 50:50 proportion of each sex is ~29°C (Wibbels 2003). The proportion of female offspring decreases from north to south due to lower sand temperature in higher latitude beaches (Marcovaldi et al. 2016). Although MSA provided valuable insights on the origin of male loggerhead sea turtles, results must be interpreted with caution due to the large confidence intervals of contribution estimates. Brazilian loggerhead sea turtle rookeries are genetically distinct from others around the world (Reis et al. 2010b;

Shamblin et al. 2014), and three distinct management units in Brazil have been suggested based on long mtDNA sequences (Shamblin et al. 2014). Although the analysis of long mtDNA fragments have refined our understanding of the genetic structure among nesting grounds, further analysis of Brazilian rookeries including larger sample sizes and other genetic markers it is necessary to better determine the boundaries of management units (Shamblin et al. 2014), as well as improve baseline data and overcome limitations of MSA (Engstrom et al. 2002; Jensen et al. 2013).

Feeding ecology and habitat use

In SIA, an important methodological issue is that lipids have low $\delta^{13}C$ values compared to other molecules, which is a potential confounding factor for stable isotope values, leading to erroneous ecological interpretations (Post et al. 2007). Skin samples of six adult males showed high lipid content capable to alter $\delta^{13}C$ values, and a defatting approach was applied. Although previous studies performed with juvenile green sea turtles indicated that skin does not have significant high lipid content (Bergamo et al. 2016), the increase in $\delta^{13}C$ values observed in our samples after lipid extraction suggests that this methodological step could be necessary to accurately determine $\delta^{13}C$ values in skin of loggerhead sea turtles. The high lipid content observed in the skin of adult males could be associated with the preferential ingestion of benthic organisms, since loggerhead prey in the neritic habitat had higher energetic values than in oceanic habitat (Barros 2010). As no significant changes in $\delta^{15}N$ values after lipid extraction with chloroform-methanol were detected, we again suggest that the extraction of lipids could be carried out from the beginning of sample processing, as a way to optimize the cost of analyses and reduce processing time in the laboratory (Medeiros et al. 2015).

Over the past decades, a foraging dichotomy between oceanic and neritic habitats has been reported for loggerhead sea turtles (Hatase et al. 2002b; Hawkes et al. 2006; McClellan et al. 2010). Our results indicated size-related differences in feeding and habitat use of male loggerheads in southern Brazil. Mixing model outputs demonstrated consistent foraging behaviour among adult males, which were shown to occur mainly in coastal waters and consume benthic organisms, preferentially the hermit crab *L. loxochelis* followed by the gastropod *B. monoliferum*. On the other hand, juvenile males showed higher variability in habitat and resource use as evidenced by the large contribution of both oceanic (i.e. salps) and neritic (the hermit crab *L. loxochelis*) to their

diet. Similarly, adult male loggerheads from the Mediterranean that were satellite-tracked showed a smaller and more neritic home range (Casale et al. 2013) than unsexed juveniles tracked in the same region (Casale et al. 2012). Also in the Mediterranean Sea, a long-term sea turtle tagging study showed a polymodal pattern of movement and habitat use among juvenile and adult loggerheads, and observed that loggerheads found in the oceanic environment are larger than turtles inhabiting neritic areas (Casale et al. 2007). In the Northwest Atlantic, adult male loggerheads of two feeding grounds showed distinct foraging strategies: while some males exhibit long-term consistency in habitat use and individual specialization, others display less site fidelity and a more variable feeding behaviour (Pajuelo et al. 2016). These patterns of temporal consistency were similar to that reported in adult female loggerheads sampled in Florida rookery, USA (Vander Zanden et al. 2010). In Brazil, satellite tracking of adult female loggerheads from the nesting population of Bahia revealed high fidelity of females to neritic foraging grounds in the northeastern (internesting) and northern (postnesting) coasts of Brazil (Marcovaldi et al. 2010). Distinct foraging strategies in adult male loggerheads were also observed in the northern Pacific (Hatase et al. 2002a; Saito et al. 2015) and in Boa Vista, West Africa (Varo-Cruz et al. 2013), and in the same way were reported to be similar to what was observed for females (Hatase et al. 2002b; Hawkes et al. 2006). The variation in foraging ecology of male loggerheads was associated with seasonal changes in sea surface temperature and with the diversity of available resources in feeding grounds, which directly affects foraging site fidelity and the degree of individual specialization in a population (Saito et al. 2015; Pajuelo et al. 2016).

The variability in the isotopic values observed in juveniles of our study could be a result of the ontogenetic habitat shift, from the oceanic to the neritic environment. In southern Brazil, it was estimated that recruitment occurs when loggerheads reach in mean 65 cm CCL (ranging from 55.7 to 77.9 cm, Monteiro 2017). Our results showed that the lowest isotopic values of males were observed in the individuals with CCL < 65 cm, indicating that skin still reflect oceanic isotopic signatures. Furthermore, previous studies reported that recruitment to neritic environments does not represent a concomitant abrupt change in habitat and diet, and that juveniles remain to feed upon oceanic-pelagic organisms for an unknown period (Barros 2010; McClellan et al. 2010). Our results also corroborate previous studies that analyzed gut contents of juvenile and adult loggerheads in southern Brazil. Although more than 45 prey have been identified, it was reported that

early juveniles using the oceanic environment consumed predominantly salps and pyrosomes, whereas large juveniles and adults foraging in neritic zones mainly prey upon hermit crabs and gastropods (Bugoni et al. 2003; Barros 2010). On the other hand, a high degree of individual specialization in resource use and a long-term fidelity to foraging grounds were observed in large juvenile of loggerheads from southern Brazil through the association of skeletochronology and SIA, and confirmed by satellite telemetry data (Monteiro 2017). Both oceanic and neritic loggerheads showed similar temporal consistency in habitat use, but the degree of individual specialization was higher in neritic turtles than in oceanic turtles. Differently from what we observed in males, this foraging polymorphism was not associated with the size of individuals, but with the variation of sea surface temperature and currents in the region (Monteiro 2017). During spring and summer, with the predominance of the tropical waters of the Brazil Current, loggerheads exhibit a greater habitat fidelity to neritic foraging grounds. However, in late autumn and winter, with the intrusion of cold waters carried by the Malvinas/Falkland Current, some loggerheads migrate to oceanic habitat while others remain close to the shore (Monteiro 2017). Although the variation in isotopic values could also be reflecting the use of different foraging areas along the coast, based on the described habitat use patterns it is unlikely to be the origin of the isotopic variation found. Nevertheless, the limited sample size of our study precludes a precise interpretation on the feeding behaviour of juvenile male loggerheads, and we recommend more extensive and targeted studies to address the variability in stable isotope values and identify possible ontogenetic shifts and polymodal foraging patterns.

In general, foraging aggregations of sea turtles are mixed stocks composed of individuals from several nesting populations (Jensen et al. 2013), and differences in diet and habitat use could be associated with the genetic variability related to such varied sources. Since male loggerheads foraging in southern Brazil have similar origins this relationship was not observed. However, there were significant differences in δ^{15}N values between male loggerheads and hybrids. Our sample size does not allow us to affirm that it is due to genetic variation. The male hybrids sampled in our study had the smallest CCL among males, and the lower δ^{15}N values could be a result of the initial life phase, in which young juveniles feed opportunistically on small pelagic organisms (Jones and Seminoff 2013). Recent studies showed the occurrence of immature hawksbill (*Eretmochelys imbricata*) and loggerhead sea turtle hybrids along the Brazilian coast, and suggested that

hybrids could be adopting ecological traits typical of loggerheads, such as feeding in southern Brazil aggregations (Proietti et al. 2014). Our results could be an indication that the same foraging and developmental ground of juvenile loggerheads is also being occupied by the offspring of loggerhead × olive ridley hybrids, but further investigations, with larger sample size and integrating genetics to SIA and telemetry, are required to address this hypothesis.

Conservation implications

The SWA represents an important foraging and development ground for loggerhead sea turtles at different life stages, and also holds extensive commercial fisheries. The significant overlap between fisheries and sea turtle distribution is currently considered the main cause of the high fishing-related mortality and the decline of several loggerhead turtle populations (Domingo et al. 2006; Wallace et al. 2013; Monteiro et al. 2016). Our findings demonstrated the importance of the foraging ground in southern Brazil for male loggerheads, mainly for Brazilian populations, which are the main stock contributors to this group. In this area, loggerhead sea turtles are highly vulnerable to bycatch in longline fisheries in the oceanic habitat (Sales et al. 2008), whereas in the neritic environment incidental capture occurs mainly in trawl and driftnet fisheries that operate along the continental shelf (Fiedler et al. 2012; Monteiro et al. 2016). The foraging dichotomy observed through SIA warns us that these individuals are exposed to both oceanic and neritic fisheries, putting the structure of populations at risk. Therefore, we strongly recommend that future fisheries management plans take into account the implementation of time-area closures in summer months in coastal areas of southern Brazil where trawling is intense, aiming to reduce the impact of fisheries on large juvenile and adult males, and bycatch mitigation measures in offshore pelagic longlines, protecting small turtles, in order to guarantee population stability and to preclude bottleneck events that could severely impact genetic diversity and survival of these populations.

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants. All applicable international, national, and institutional guidelines for the care and use of animals were followed. Sampling was conducted under SIBIO licenses Nos. 15962-5 and 49019-1 to 49019-3 (SISBIO- *Sistema de Autorização e Informação em Biodiversidade)*.

No live animals were used for experiments or sampling. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Table 1 Standard diversity indices (mean ± standard deviation) calculated for male loggerhead sea turtles in southern Brazil with short and long sequences. *N* corresponds to the number of haplotypes, *h* is haplotype diversity, and π is nucleotide diversity

Sequence size	N	h	π
380pb	2	0.083 ± 0.075	0.003 ± 0.003
818pb	4	0.543 ± 0.085	0.003 ± 0.002

Table 2 Estimated contributions of Brazilian loggerhead rookeries to the male aggregation at the southern Brazil foraging ground, based on Bayesian Markov Chain Monte Carlo mixed stock analysis. Mean values are shown with standard deviation (SD). The 2.5 and 97.5% values indicate the upper and lower bounds of the 95% credibility interval

Stock	Mean	SD	2.5%	Median	97.5%
Sergipe	0.2399	0.1371	0.1325	0.2386	0.5102
Bahia	0.2476	0.1358	0.1439	0.2494	0.5150
Espírito Santo	0.2619	0.1228	0.1709	0.2555	0.5180
Rio de Janeiro	0.2506	0.1310	0.1508	0.2553	0.5230

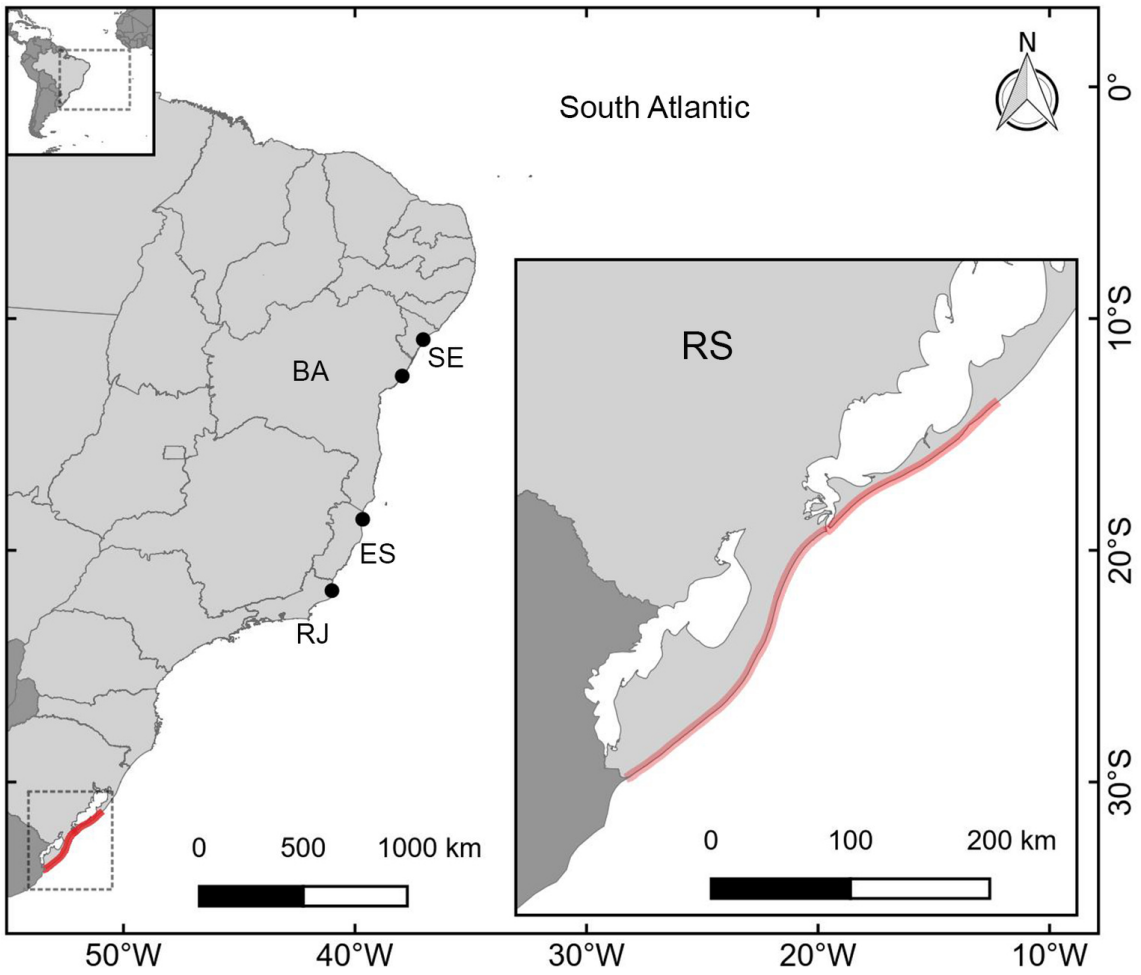

Fig. 1 Map of the Brazilian coast, with black dots indicating the main loggerhead sea turtle nesting sites in Brazil, located in the states of Sergipe (SE), Bahia (BA), Espírito Santo (ES) and Rio de Janeiro (RJ). Zoomed map indicates the study area along the Rio Grande do Sul (RS) state coastline, in southern Brazil, with red line indicating sampling location

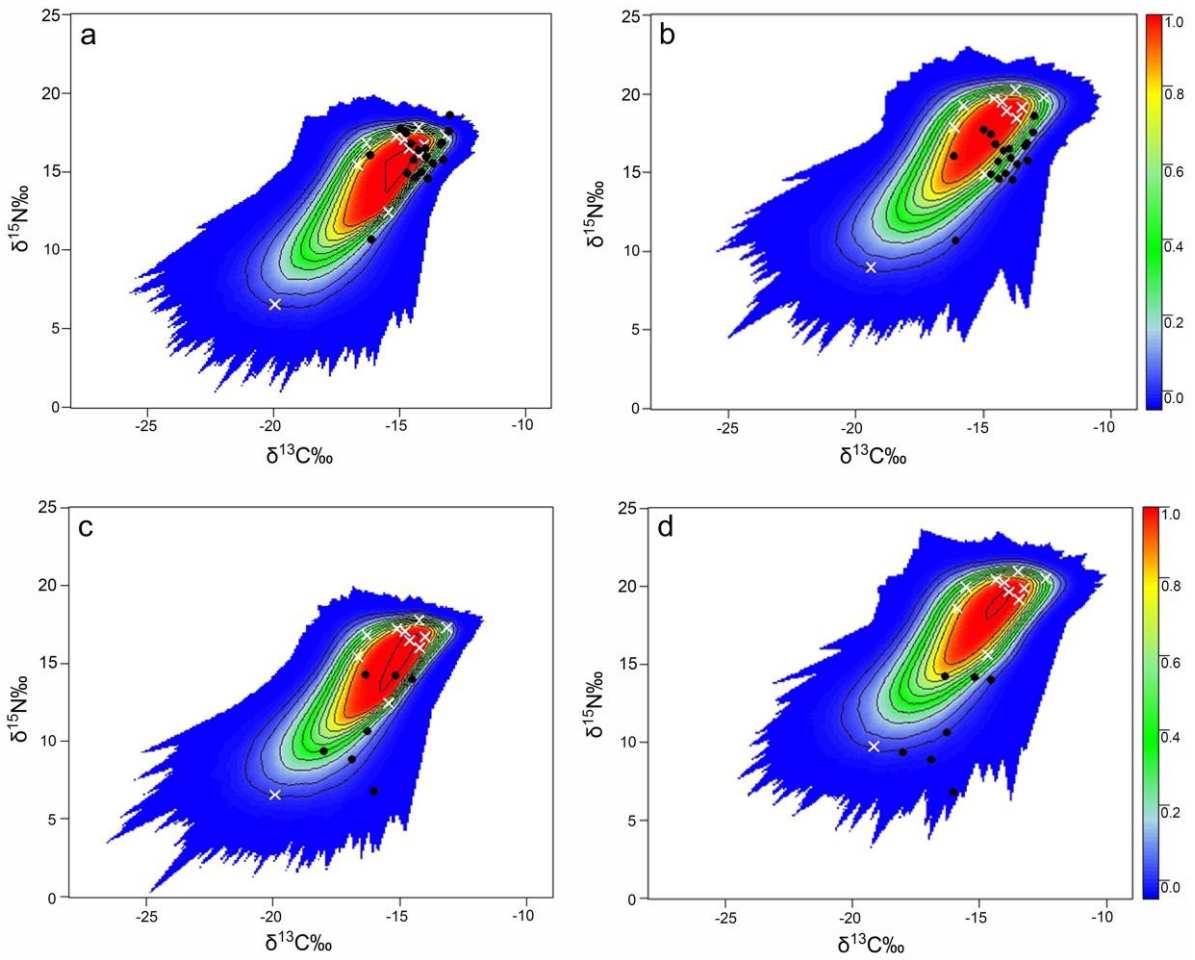

Fig. 2 Simulated mixing models for male loggerhead sea turtles from southern Brazil using three different sets of trophic discrimination values for correcting prey isotopic values. For adults: (a) 1.11 ± 0.17‰ for δ^{13}C and 1.60 ± 0.07‰ for δ^{15}N (Reich et al. 2008), and (b) 1.62 ± 0.61‰ and 4.04 ± 0.044‰ respectively for δ^{13}C and δ^{15}N (Vander Zanden et al. 2012); for juveniles: (c) 1.11 ± 0.17 ‰ for δ^{13}C and 1.60 ± 0.07‰ for δ^{15}N (Reich et al. 2008), and (d) 1.87 ± 0.56‰ for δ^{13}C and 4.77 ± 0.40‰ for δ^{15}N (Vander Zanden et al. 2012). Position of the consumers (black dots) and the average source signatures (white crosses) are shown. Probability contours (black lines) are at the 5% level (outermost line) and successively at each 10% level

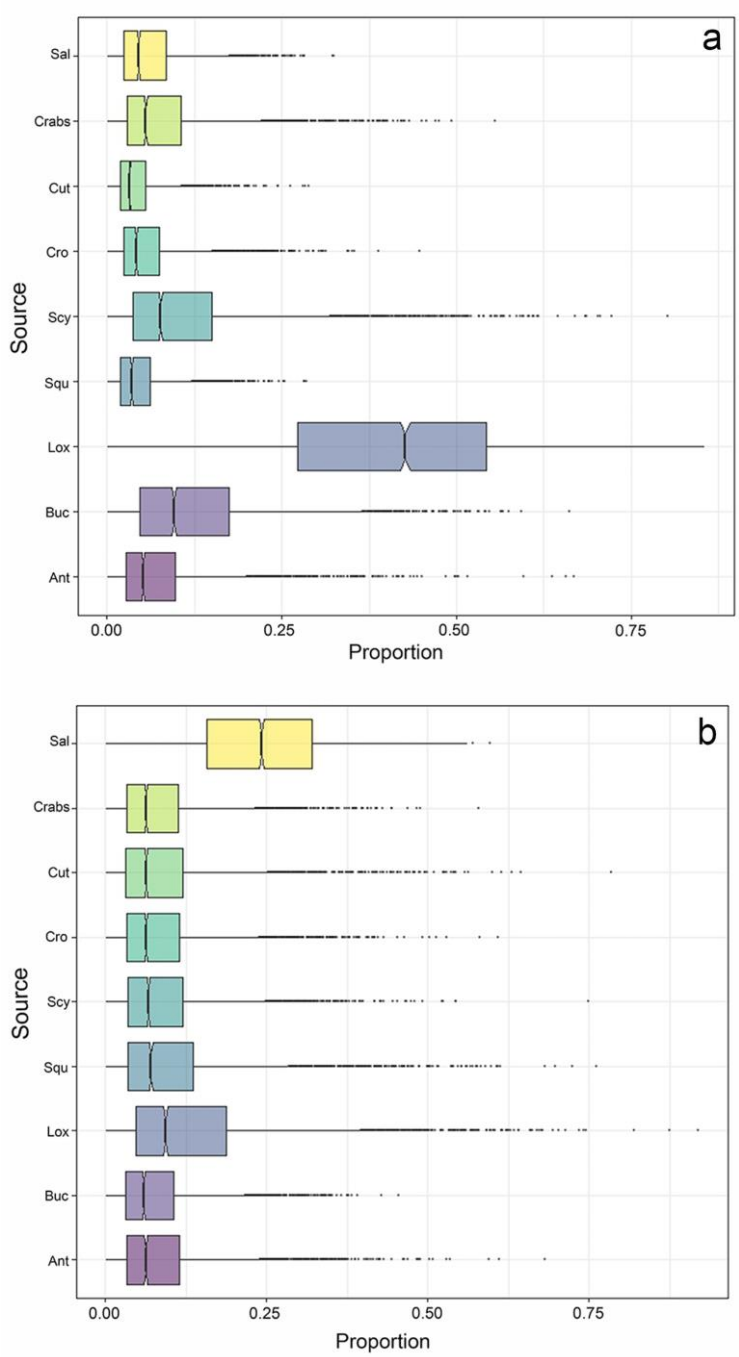

Fig. 3 Results of Bayesian Stable Isotope Mixing Models showing estimated prey contributions (mean, 25% and 75% percentiles) to the diets of (a) adult and (b) juvenile male loggerhead sea turtles. Sal – Salps, Cra – Crabs (i.e. *Libinia spinosa* and *Dardanus insignis*), Cut – Cutlassfish (*Trichiurus lepturus*), Cro – Croakers (i.e. white croaker *Micropogonias furnieri* and banded croaker *Paralonchurus brasiliensis*), Scy – Schyphozoa (i.e. jellyfish *Lychnorhiza lucerna*), Squ – Squid (*Dorytheuthis plei*), Lox – *Loxopagurus loxochelis*, Buc – gastropod *Buccinanops monoliferum*, Ant – Anthozoa (i.e. anemones)

Supplementary material

Table S1 Male loggerhead sea turtle *Caretta caretta* dataset. CCL is curved carapace length measured in centimeters; D is the decomposition state of carcass, as follows:1 = freshly dead, 2 = initial decomposition, 3 = moderate decomposition, 4 = intermediate decomposition, and 5 = advanced decomposition; $\delta^{13}C$ and $\delta^{15}N$ are stable isotope values of carbon and nitrogen in parts per thousand (‰), respectively; and C:N is weight percent carbon to nitrogen concentrations ratios. *Values after lipid extraction. •Individuals excluded from Stable Isotope Mixing Models. NA = not available

Turtle ID	Date	CCL	D	Life stage	Haplotype	$\delta^{15}N$	$\delta^{13}C$	C:N
RSE001	February 24, 2015	92.5	4	Adult	CC-A4.2	14.97	-14.12	3.0
RSE002	February 05, 2014	114	3	Adult	CC-A4.2	16.83	-13.30	3.4
RSE003	December 16, 2014	74.5	3	Juvenile	CC-A4.1	14.26	-16.36	3.5
RSE006•	April 07, 2015	107.5	3	Adult	CC-A4.2	15.77	-13.24	3.2
RSE007	December 16, 2014	98.0	3	Adult	CC-A4.2	15.73	-14.42	3.1
RSE008	January 13, 2015	99.0	3	Adult	CC-A4.1	10.69	-16.10	2.9
RSE009	February 10, 2014	106.0	4	Adult	CC-A4.2	14.53	-13.86*	2.9*
RSE010	December 09, 2014	97.0	3	Adult	CC-A4.2	15.92	-13.92	3.0
RSE011	March 07, 2014	NA	4	Adult	CC-A4.2	17.42	-14.71	3.2
RSE012	March 17, 2016	100.0	4	Adult	CC-A4.2	17.74	-14.97	3.5
RSE013	March 17, 2016	101.0	4	Adult	CC-A4.2	16.38	-14.22*	3.2*
RSE014	December 28, 2015	90.0	3	Adult	CC-A4.2	15.53	-13.67*	2.9*
RSE015	February 18, 2016	98.0	5	Adult	CC-A4.1	16.50	-13.99*	2.9*
RSE017	December 09, 2016	40.0	3	Juvenile	CC-A4.2	9.38	-18.01	3.5
RSE019	March 30, 2016	98.6	4	Adult	CC-A4.1	16.06	-16.14	3.4
RSE020	February 21, 2017	101.5	4	Adult	CC-A4.2	16.70	-13.40	3.0
RSE021	February 21, 2017	101.0	4	Adult	CC-A4.1	18.60	-12.99*	2.7*
RSE029	February 27, 2017	96.0	3	Adult	CC-A4.2	14.60	-14.40	3.3
RSE030	February 27, 2017	98.0	3	Adult	CC-A4.1	14.90	-14.70	3.2
RSE033	December 28, 2016	105.0	3	Adult	CC-A4.3	16.81	-14.53	3.3
RSE047	March, 01, 2017	61.5	4	Juvenile	Cc × Lo	8.86	-16.91	3.1
RSE049	March 01, 2017	101.0	4	Adult	CC-A4.2	17.54	-13.03*	2.8*
RSE054•	February 25, 2014	24.0	1	Juvenile	Cc × Lo	6.80	-16.03	2.8
RSE067	July 31, 2015	78.0	1	Juvenile	CC-A4.2	14.00	-14.54	3.0
RSE084	March 17, 2016	62.3	1	Juvenile	CC-A2.1	14.20	-15.20	2.8
RSE113	January 29, 2016	64.2	1	Juvenile	CC-A4.1	10.62	-16.28	2.9

Table S2 Summary of GLM results of $\delta^{13}C$ and $\delta^{15}N$ in relation to haplotype and size class. Bold indicates categories of explanatory variables used as references in the analysis. SE = standard error

Model	Parameters	Estimate	SE	*t*	*P*
$\delta^{13}C$~ Haplotype + Size class					
	Intercept	-13.213	1.108	-11.923	< 0.001
	Adult	–	–	–	–
	Juvenile	-1.976	0.543	-3.638	< 0.001
	CC-A2.1	–	–	–	–
	CC-A4.1	-1.443	1.1032	-1.308	0.20571
	CC-A4.2	-0.772	1.1032	-0.700	0.49211
	CC-A4.3	-1.3162	1.4702	-0.895	0.3813
	Cc × Lo	-1.28	1.1831	-1.082	0.29217
$\delta^{15}N$~ Haplotype + Size class					
	Intercept	17.947	2.14	8.385	< 0.001
	Adult	–	–	–	–
	Juvenile	-3.747	1.049	-3.572	< 0.001
	CC-A2.1	–	–	–	–
	CC-A4.1	-2.358	2.131	-1.107	0.28152
	CC-A4.2	-1.912	2.131	-0.897	0.38024
	CC-A4.3	-1.137	2.839	-0.401	0.69296
	Cc × Lo	-6.37	2.285	-2.788	< 0.001

Table S3 Mean ± standard deviation of $\delta^{15}N$ and $\delta^{13}C$ values of potential prey of *Caretta caretta* at the study region. *n* = number of individuals

Taxonomic group/species	*n*	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)
Anthozoa	2	14.88 ± 0.24	-15.71 ± 0.18
Schyphozoa			
Lychnorhiza lucerna	3	14.40 ± 0.17	-15.32 ± 0.10
Gastropoda			
Buccinanops monoliferum	5	15.71 ± 0.39	-14.26 ± 0.35
Cephalopoda			
Dorytheuthis plei	4	13.83 ± 0.53	-17.75 ± 0.50
Crustacea			
Dardanus insignis	4	16.19 ± 0.40	-15.34 ± 0.18
Loxopagurus loxochelis	2	10.82 ± 0.22	-16.54 ± 0.10
Libinia spinosa	6	15.12 ± 0.23	-15.11 ± 0.34
Thaliacea	8	4.95 ± 1.56	-21.03 ± 1.59
Actiopterigii			
Micropogonias furnieri	17	15.46 ± 0.62	-15.89 ± 0.45
Paralonchurus brasiliensis	15	15.64 ± 0.44	-16.21 ± 0.63
Trichiurus lepturus	15	15.21 ± 0.93	-17.41 ± 0.70

CAPÍTULO 3

Population structure and bottleneck analyses of loggerhead sea turtles *Caretta caretta* in the southwestern Atlantic Ocean

Luciana Medeiros, Maíra C. Proietti, Eduardo R. Secchi

Population structure and bottleneck analyses of loggerhead sea turtles *Caretta caretta* in the southwestern Atlantic Ocean

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Abstract

The southwestern Atlantic Ocean (SWA) harbors one of the largest nesting aggregations of loggerhead sea turtles (*Caretta caretta*). Although the abundance of this species is currently considered stable or increasing, past reductions due to human activities could have resulted in a variety of genetic and demographic processes that lead to losses in genetic diversity and reductions in fitness. The present study investigated the genetic structure and the occurrence of population bottleneck in loggerhead sea turtle from nesting grounds in SWA. Nine microsatellite markers were assessed in 100 samples from four nesting sites in Brazil. No significant genetic differentiation was observed among nesting areas, indicating male-mediated gene flow that increases nuclear genetic variability and maintains population stability. No deviation from mutation-drift equilibrium was detected, but M-ratio values indicted that population bottleneck events occurred. Although population bottleneck analyses indicate that the population is generally in equilibrium, there are indications that the loggerhead population in SWA has experienced a drastic reduction in last generations that should be considered in the implementation and/or maintenance of conservation measures.

Introduction

The knowledge of the conservation status and viability of sea turtle populations is challenging due to logistic and financial difficulties in conducting systematic surveys in marine ecosystems (Seminoff et al. 2014). Currently, estimates of population abundance used for sea turtles is based on annual counts of nesting females throughout each reproductive season, and the increase in nests over the last decade is assumed to be an indication of increase in population size (Casale and Tucker 2017). However, since sea turtles are slow-growing and late-breeding animals (Avens et al. 2015), this method is not an appropriate representation of the effective population size (Casale and Tucker 2017), as this estimate is based only on a portion of the population and does not take into account human-induced removal of individuals to be recruited to colonies, as adult males and large juveniles (Heppel 1998, Peckham et al. 2011).

Significative reductions in population abundance lead to a variety of genetic and demographic processes that can hasten extinction due to losses in genetic diversity and reduction in fitness (Frankham et al. 1999, Allendorf et al. 2001). One of the consequences of abrupt population size decrease is the bottleneck effect, characterized by an increase in inbreeding and genetic drift, and reduction in genetic variation (Frankham et al. 1999). Populations that have recently undergone population bottleneck events have rare allele deficiencies, and it is possible to detect this scenario through tests that consider allelic frequency distribution and excess of heterozygosity in relation to the total number of alleles (Cornuet and Luikart 1996, Peery et al. 2012). A previous genetic study using microssateilte markers showed a clear signal of bottleneck event in six of 13 analyzed olive turtle (*Lepidochelys olivacea*) populations from Mexico, revealing a pattern consistent with the demographic imbalance caused by decades of intense commercial exploitation (Rodríguez-Zárate et al. 2013). For loggerhead sea turtle (*Caretta caretta*), signals of recent population bottleneck were reported in rookeries in Cyprus and Turkey, in the Mediterranean Sea, but no potential human impact was suggested in this case (Clusa et al. 2018). On the other hand, despite the intense slaughter of females and males, egg harvesting and bycatch of loggerheads around the Cape Verde Islands, eastern Atlantic, no bottleneck signal was detected in the nuclear DNA (nDNA) of nesting females (Monzón-Argüello et al. 2010). These results contrast with the high mortality rate in the region and indicate that the current scenario has not yet compromised the genetic diversity of the loggerhead populations.

In the southwestern Atlantic Ocean (SWA), the loggerhead sea turtle has also a long history of threats. Before the 1980s, most of the eggs were collected and many females were slaughtered for food by local communities of nesting grounds in Brazil (Marcovaldi and Marcovaldi 1999, Silva et al. 2016). From this decade on, with the creation and establishment of Projeto TAMAR, a sea turtle conservation-oriented project in the main nesting grounds of sea turtles began to contribute to the recovery of Brazilian loggerhead populations, with a significant increase in the abundance of nesting females and number of nests per season (Marcovaldi and Chaloupka 2007, Marcovaldi et al. 2018). Currently, this region hosts one of the largest nesting aggregation of loggerhead sea turtles worldwide (ca. 7000 to 8000 nests/year; Marcovaldi et al. 2018), with rookeries ranging over a wide latitudinal area along the Brazilian coastline, from Sergipe to Rio de Janeiro states (Marcovaldi and Chaloupka 2007). A phylogeographic study based on the mitochondrial DNA (mtDNA) control region proposed that Brazilian rookeries encompass three distinct management units (MUs – *sensu* Moritz 1994), holding endemic haplotypes: (1) the northeastern coast (Sergipe and Bahia states), (2) Espírito Santo, and (3) Rio de Janeiro (Fig. 1, Shamblin et al. 2014); such distinctions arise in sea turtle populations due to their natal phylopatric behavior. At a global scale, the MUs of sea turtles have been established based on mtDNA haplotype frequencies reported in rookeries across ocean basins. Nevertheless, since this genetic marker is maternally inherited, further studies using nuclear DNA markers (nDNA), such as microsatellites, are necessary to confirm the population structure and distribution of MUs in SWA, and consequently to help assess the conservation status of sea turtles in this region (Shamblin et al. 2014, Casale and Tucker 2017).

Over the past decades, other anthropogenic threats began to significant impact sea turtle survival in the SWA, such as coastal development (Lopez et al. 2014, Silva et al. 2016), marine pollution (Rizzi et al. 2019) and, most of all, bycatch in fisheries in foraging grounds (Sales et al. 2008, Fiedler et al. 2012, Monteiro et al. 2016). Genetic studies with mtDNA revealed that neritic foraging grounds of loggerhead turtles in the SWA are composed exclusively by individuals from Brazilian rookeries (Caraccio et al. 2008, Prosdocimi et al. 2015), whereas oceanic feeding aggregations also harbor loggerheads from Indo-Pacific and the North and East Atlantic (Caraccio et al. 2008, Reis et al. 2010, Shamblin et al. 2014). However, information on the effects of all these threats on the demographic and genetic structure of populations from the SWA is understudied, and the

limited available data on population abundance of loggerhead sea turtle preclude the assessment of significant events of population decline over the last generations (Santos et al. 2011).

In this context, the aim of the present study was to assess the population structure of Brazilian nesting grounds and evaluate whether decades of threats have led to population bottleneck events, increasing inbreeding and causing loss of genetic diversity of loggerhead turtles in the SWA.

Methods

Sample collection

Skin samples were collected during nesting seasons between 2004 and 2018 from 79 female loggerhead at rookeries in Brazil (Fig. 1): Sergipe (n = 8), Bahia (n = 21), Rio de Janeiro (n = 31) and Espírito Santo (n = 19). Additionally, 21 hatchlings were sampled from independent nests in Bahia. For nesting females, curved carapace length (CCL) was measured with a flexible metric tape (± 0.1 cm), from the mid-point of the nuchal scute to the posterior end of the posterior marginal scute (Bolten 1999). Hatchlings had their straight carapace length (SCL) measured with calipers (± 0.1 mm). The mean CCL of nesting females was 99 ± 0.7 cm (range 83-117 cm), and the mean SCL of hatchlings was 4.1 ± 0.2 cm (3.9-4.5 cm). Tissue samples were collected using 6-mm biopsy punch, stored in sodium chloride (NaCl) or absolute ethanol, and frozen at -20°C until laboratory procedures.

Microsatellite analysis

Nine previously described microsatellite loci for sea turtles were amplified: Cc117, Cm72 and Cm84 (FitzSimmons et al. 1995); Cc7 and Cc141 (Bowen et al. 2005); Ccar176 (Carreras e al. 2007); Cc17, Cc25 and Cc28 (Monzón-Argüello et al. 2008). One primer of each marker was fluorescently labelled with 6-FAM or HEX dyes incorporated by M13 tail (Schuelke 2000). Polymerase Chain Reactions (PCR) contained 20 to 50 ng of genomic DNA, 1.5U of Platinum Taq Polymerase, 0.1 µM forward primer, 0.2 µM reverse primer, 0.2 µM fluorescently labelled M13 primer, 0.4 mM of dNTPs, 10x PCR Buffer and 2.5 mM $MgCl_2$. Negative controls were included to detect possible

contaminations during the amplification process. PCRs were performed with an Applied Biosystems Veriti 96-well Thermocycler. For loci Cc117, Cm72, Cm84, Cc7, Cc141, reactions were carried out using the following thermal cycling program: an initial denaturation of 5 min at 94°C; 30 cycles of 30 s at 94ºC, 1 min at 53ºC, 45 s at 72ºC; and a final extension of 10 min at 72ºC. For loci Cc17, Cc25, Cc28 and Ccar176, reactions were conducted using a cycle of an initial denaturation of 5 min at 94°C; 20 cycles of 30 s at 94ºC, 30 s at 61ºC (56ºC for locus Ccar176), 30 s at 72ºC; 15 cycles of 30 s at 94ºC, 45 s at 53ºC, 45 s at 72ºC; and a final extension of 10 min at 72ºC. Fragment analysis were conducted on ABI 3730XLs automated sequencer with an internal standard size marker of 400HD. About 10% of the samples were genotyped twice independently at all loci to estimate scoring error rate (Bonin et al. 2004).

Data analysis

Allele sizes were assigned with Peak Scanner™ v.2 (Applied Biosystems). Null alleles, large allele dropout and scoring errors were assessed in MICRO-CHECKER (Oosterhout et al. 2003). Deviations from Hardy-Weinberg equilibrium and linkage disequilibrium between loci pairs were estimated using Genepop v. 4.5 (Rousset 2008). Allelic richness was estimated in FSTAT 2.9.3 (Goudet 2001). The mean observed (H_o) and expected (H_e) heterozygosities, mean number of alleles for each locus, inbreeding indices (F_{is}), and pairwise genetic distances (F_{st}) were calculated using ARLEQUIN 3.1 (Excoffier et al. 2005). Pairwise genetic distances D_{st} were estimated using POPTREEW (Takezai et al. 2014). A Mantel test was performed in R 3.4.2 (R Core Team 2017) to detect possible isolation by geographical distance between nesting grounds. Furthermore, a Bayesian clustering method was applied to estimate the most likely number of population (K) within the study area through STRUCTURE 2.3.4, assuming as informative prior that each rookery are distinct genetic units (Pritchard et al. 2000). The parameter K varied from 1 to 8; for each K, 20 runs and 100,000 Markov Chain Monte Carlo (MCMC) interactions were simulated with an initial burn-in of the first 10,000. The best K was estimated with the mean likelihood of simulations and the ad hoc ΔK *ad hoc* statistic in STRUCTURE HARVESTER (Evanno et al. 2005). The 20 runs of the best K were combined in CLUMPP v.1.1.2 (Jakobsson and Rosenberg 2007) and the final result generated by DISTRUCT v. 1.1 (Rosenberg, 2004).

Potential recent population bottlenecks were assessed using three distinct analytical methods: frequency of rare alleles in the population (Luikart et al. 1998), heterozygosity excess (Cornuet and Luikart 1996) and *M*-ratio (Garza and Williamson 2001). The first method graphically plots the distribution of allele frequencies, showing the proportion of rare alleles in the population: L-shaped distributions indicate that the population is in mutation drift-equilibrium, whereas asymmetric shapes indicate a recent bottleneck event (Luikart et al. 1998). The second approach tested for heterozygosity excess with the Wilcoxon sign-rank test, under the assumption of a two-phase mutation model (TPM), with the variance among multiple steps set at 22 and the proportion of multi-step mutations set at 57%, as estimated for green turtle *Chelonia mydas* (Peery et al. 2012) in BOTTLENECK v.1.2.02 (Piry et al. 1999). The third method consists of the ratio between the total number of alleles and the range of allele size + 1 (Garza and Williamson 2001). It is assumed that, for allele matrices with ≥ 7 loci, *M*-ratios lower than 0.68 indicate that the population has undergone abrupt decline and a bottleneck event (Garza and Williamson 2001). Genetic diversity indices and bottleneck analyses were performed at two levels: (1) grouping all SWA populations and (2) separately for each rookery (deme).

Results

A total of 80 alleles across the microsatellite loci were recorded, ranging from 5 to 12 (Table 1). All microsatellite loci were variable, with an average of 8.87 alleles per locus, mean observed heterozygosity of 0.70 and allelic richness of 8.77 (Table 2). Independence of loci was assumed, as no linkage disequilibrium was observed between loci pairs (χ^2 test, $P > 0.05$ in all cases). The locus Cc25 deviated from Hardy-Weinberg equilibrium in almost all nesting grounds, with the exception of the Sergipe rookery, and was removed from further population analysis. Inbreeding coefficient values were negative for all rookeries, ranging from -0.2634 to -0.1107 (Table 2). Pairwise genetic distance index F_{st} ranged from -0.044 to 0.007, and D_{st} from -0.005 to 0.049, revealing that there are non-significant differences among nesting areas (Table 3). The Mantel test was also non-significant, showing no correlation between genetic and geographical distances (Fig. 2). Bayesian analysis of structure resulted in $K = 2$ as the most likely number of populations (mean ln likelihood = -2858.54, SD = 8.15, $\Delta K = 14.44$), indicating genetic differences between Sergipe/Espírito Santo and Bahia/Rio de Janeiro (Fig. 3).

Evaluation of allele frequency distributions showed a large number of rare alleles across nesting grounds, with most rookeries displaying L-shaped distribution curves except the Sergipe rookery, which showed an asymmetric shape (Fig. 4). The Wilcoxon test for population bottleneck based on TPM did not reveal significant heterozygote excess in Brazilian loggerhead nesting sites, indicating that all stocks were in mutation-drift equilibrium (Table 2). M-ratio results ranged from 0.42 ± 0.17 to 0.60 ± 0.17, showing values below the critical value of 0.68 and indicating recent population bottleneck events in the SWA rookeries (Table 2).

Discussion

The Brazilian loggerhead nesting populations are one of the largest in the world and are known to hold endemic haplotypes delineated by maternal inheritance (Reis et al. 2010, Shamblim et al. 2014). For the first time, a complementary investigation on the genetic differentiation between these rookeries was carried out using nDNA markers. Genetic distance values of nuclear markers showed no differentiation and no distance-related structure among nesting grounds, differently from what has been observed with mtDNA for the region (Reis et al. 2010, Shamblin et al. 2014). Previous genetic analysis of mtDNA of loggerhead turtles from North Atlantic rookeries also reported that the natal philopatric behavior of females results in high genetic structuring among nesting grounds, resulting in at least six management units at the region (Shamblin et al. 2014). Significant male-mediated gene flow may explain the lack of population structure in nuclear markers relative to mitochondrial markers, promoting exchange of genes among populations (Bowen et al. 2005, Jensen et al. 2013, but see Clusa et al. 2018). On the other hand, studies in the Mediterranean Sea using microsatellite markers detected high genetic differentiation among loggerhead rookeries, similar to what was observed with mitochondrial markers, indicating that both females and males display philopatric behavior (Carreras et al. 2007, Clusa et al. 2018). In the eastern Atlantic, loggerhead rookeries from Cape Verde did not show structuring among nesting grounds neither by mtDNA nor nDNA, although the region was recognized as significantly distinct from other Atlantic and Mediterranean nesting areas (Monzón-Argüello et al. 2010).

The two populational clusters observed in ΔK statistic did not corroborate previous mtDNA-based management units proposed for the SWA rookeries (Reis et al. 2010, Shamblin et al. 2014), or the pairwise genetic distance and Mantel test applied in the

present study. Therefore, caution is required when considering this clustering result. Although ΔK statistics usually estimate the correct number of clusters in most situations, it should not be used exclusively, because this method is unable to identify the best K if $K = 1$, i.e., if the sampled areas correspond to one single population (Evanno et al. 2005), which is possibly the case in the present study with microsatellites. Furthermore, the analysis is sensitive to the number of loci analyzed and sampled localities as well as the number of individuals in each area (Evanno et al. 2005), and the results presented here could be reflecting the low sample size in Sergipe and Espírito Santo and the unamplified loci of some individuals in these locations.

High genetic variability was observed on Brazilian nesting grounds, with a high proportion of rare alleles, with the exception of Sergipe rookery. In this locality, the allelic frequency distribution showed a shifted mode, indicating an uneven proportion of alleles that likely corresponds to drastic reductions in abundance. However, this inference seems to be influenced by the sample size in this location, which was insufficient to detect the real allelic frequency of the rookery. Nevertheless, further studies with larger sample sizes are required to better assess the genetic diversity of this rookery.

The identification of genetic bottlenecks is fundamental to establish management practices aimed at restoring population connectivity, and minimize additional impacts caused by human activities. The Wilcoxon rank test did not detect excess of heterozygosity in any nesting area. However, *M-ratio* values detected loss of genetic diversity in all rookeries (Garza and Williamson 2001), confirming that the past reductions in population sizes have reduced diversity. Populations that have suffered drastic reductions show an excess of heterozygosity for only a few generations due to the high mutation rate of nuclear marker (Luikart et al. 1998). *M-ratio* values, however, can remain low for up to 50 generations after the bottleneck, because mutation rates do not rapidly increase the number of alleles at the same proportion as allele size range (Garza and Williamson 2001). Therefore, the population bottleneck signal observed here does not appear to be associated with the historically reported threats and high mortalities that occurred in the SWA over the past two generations (since the late 20th century), but with older bottleneck events, since populations were in mutation drift-equilibrium (Piry et al. 1999).

Recently, population bottlenecks have been identified through nuclear markers in the Cyprus and Turkey loggerhead rookeries in the Mediterranean Sea, but were attributed

to recent colonization of western Mediterranean (Clusa et al. 2018). In the eastern Atlantic, despite the high mortality rate of loggerheads in Cape Verde islands caused by ongoing fisheries and the intense consumption of eggs and adult turtles, no bottleneck event was detected in the population, indicating that the current exploitation scenario has not yet compromised the genetic diversity of the population (Monzón-Argüello et al. 2010). In this region, a previous study revealed that directional male-mediated gene flow sustains the adaptive potential of the entire rookery, ensuring the maintenance of genetic diversity and potentially compensating population reductions (Stiebens et al. 2013). In the SWA, the threat history on loggerhead sea turtles is similar to that reported in Cape Verde and it seems that male behavior is also promoting gene flow and maintaining the genetic stability of the population. This information is important for directing conservation measures, especially in areas with aggregations of male loggerheads. Southern Brazil was recently recognized as an important feeding grounds for male loggerheads of Brazilian lineages (Medeiros et al. 2019). In this area, they are highly vulnerable to bycatch in fisheries in the oceanic and neritic habitats (Monteiro et al. 2016). Therefore, it is crucial that management plans take into account these feeding grounds in order to maintain the stability of the Brazilian population and to avoid severely impacts on genetic diversity.

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Table 1. Information summary of the nine microsatellite loci amplified in loggerhead sea turtles from nesting grounds in Brazil. Size = allele size in base pairs; *K* = number of alleles; *Ho* = observed heterozygosities; *He* = expected heterozygosities. *Locus with significant deviation from Hardy-Weinberg equilibrium ($P< 0.05$).

Locus	Size	*K*	*Ho*	*He*
Cc17	340-374	11	0.57732	0.57849
Cc25*	334-352	9	0.96774	0.72688
Cc28	202-230	10	0.90526	0.80791
Cc141	205-229	8	0.75000	0.72648
Cm84	330-352	8	0.67059	0.59192
Cm72	240-266	5	0.43373	0.43834
Cc7	182-212	9	0.53409	0.58545
Cc117	248-266	8	0.85263	0.76486
Ccar176	195-225	12	0.93617	0.84003

Table 2. Genetic diversity summary statistics for loggerhead sea turtle nesting grounds in Brazil. *n* = sample size; *Na* = mean number of alleles; *Ar* = mean allelic richness; *Ho* = mean observed heterozygosities; *He* = mean expected heterozygosities; F_{is} = inbreeding coefficient; *P* = probability of detecting a bottleneck in rookeries with Wilcoxon rank test under the two-phase mutation model; *M-ratio* = ratio of number of alleles by range of allele size + 1.

Location	*n*	*Na*	*Ar*	*Ho*	*He*	F_{is}	*P*	*M-ratio*
Sergipe	8	3.750 ± 1.392	3.1538 ± 1.02	0.6302 ± 0.228	0.6165 ± 0.180	-0.1835	0.2128	0.42 ± 0.17
Bahia	42	7.875 ± 1.833	3.6500 ± 0.85	0.7226 ± 0.162	0.6810 ± 0.111	-0.1107	0.9755	0.60 ± 0.17
Espírito Santo	19	5.875 ± 1.536	3.5348 ± 0.949	0.6983 ± 0.235	0.6450 ± 0.191	-0.2634	0.6328	0.57 ± 0.23
Rio de Janeiro	31	6.375 ± 2.118	3.4442 ± 1.030	0.7014 ± 0.180	0.6505 ± 0.150	-0.1400	0.7519	0.56 ± 0.17
All colonies	100	8.875 ± 2.027	8.7780 ± 2.089	0.7074 ± 0.172	0.6666 ± 0.130	-0.1559	0.2803	0.65 ± 0.14

Table 3. Pairwise genetic distances between Brazilian loggerhead sea turtle rookeries. F_{st} values are below the diagonal and D_{st} values above the diagonal. All values were non-significant.

	Sergipe	Bahia	Espírito Santo	Rio de Janeiro
Sergipe		0.049	0.040	0.044
Bahia	-0.025		-0.005	0.013
Espirito Santo	-0.015	-0.044		0.028
Rio de Janeiro	-0.013	0.007	-0.026	

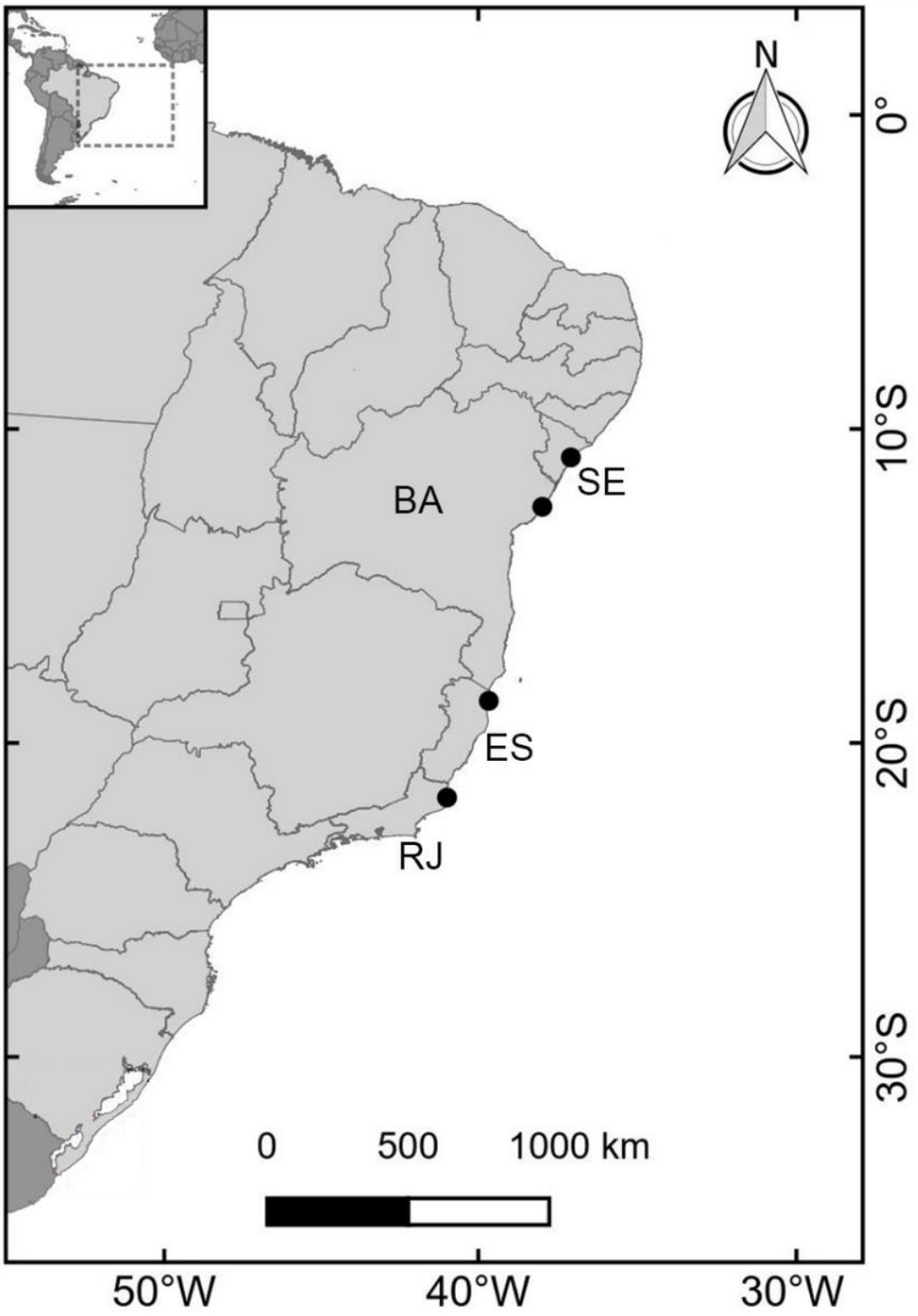

Fig. 1. Map of loggerhead sea turtle rookeries along coastline of Brazil, in the southwestern Atlantic Ocean. Abbreviations indicates Brazilian states where nesting grounds are located. SE= Sergipe, BA= Bahia, ES= Espírito Santo, RJ= Rio de Janeiro.

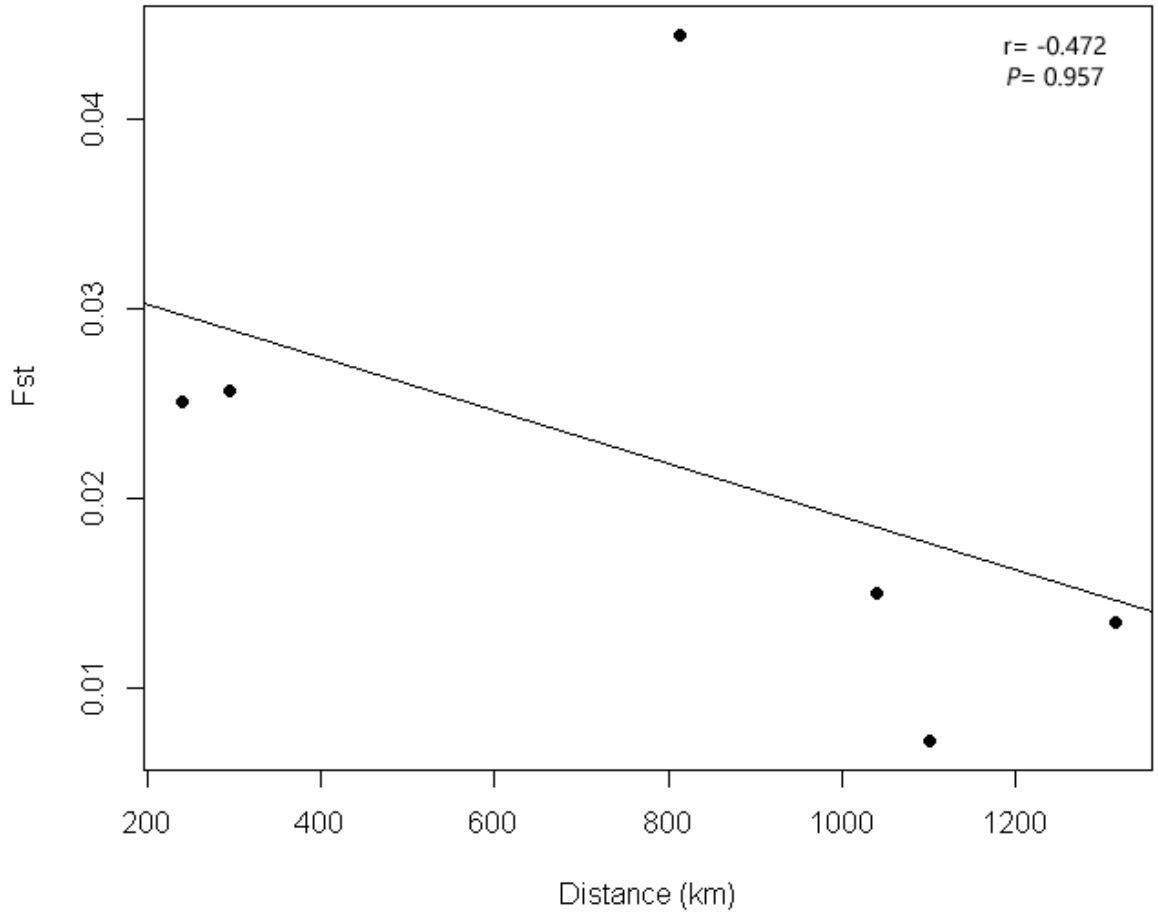

Fig. 2. Linear regression between F_{st} values and shortest distance (km) along the coastline of nesting grounds in Sergipe, Bahia, Espírito Santo and Rio de Janeiro, Brazil.

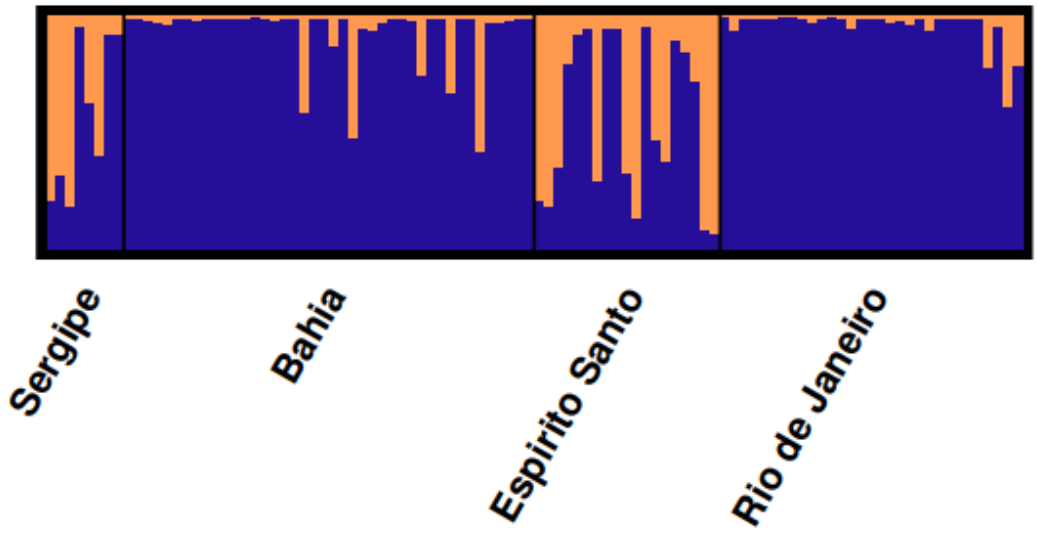

Fig. 3. Estimated probabilities of each individual loggerhead sea turtle to each cluster identified by STRUCTURE ($K = 2$) in four Brazilian nesting grounds.

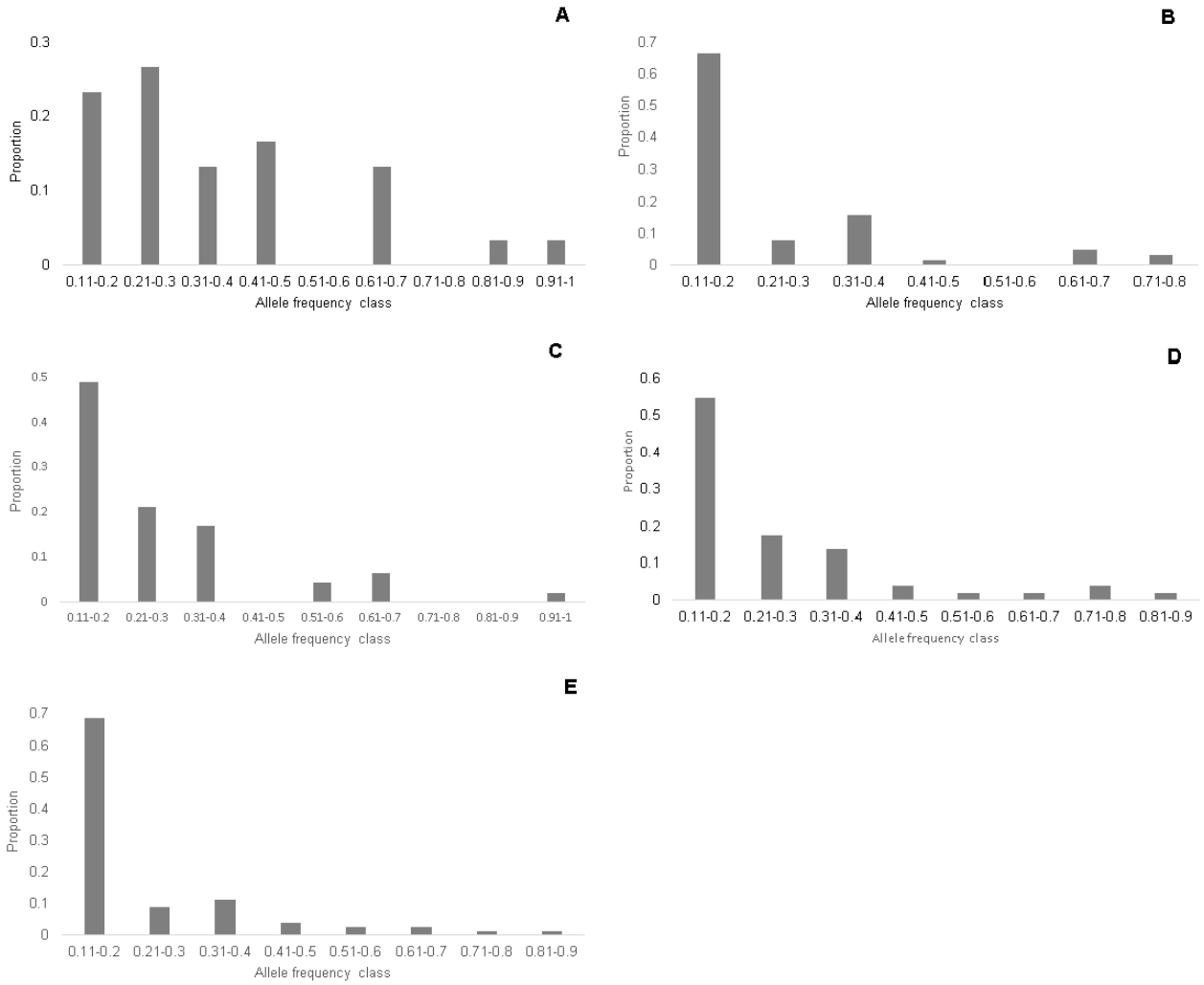

Fig. 4. Allele frequency distributions of loggerhead sea turtles from Brazilian rookeries: (A) Sergipe, (B) Bahia, (C) Espírito Santo, (D) Rio de Janeiro, and (E) All colonies.